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

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Abstract
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, Pro-1 is a general base that abstracts the 2-hydroxyl proton of 1 for delivery to the C-5 position to yield 2. In the present study, 4-OT was explored for nucleophilic catalysis based on the mechanistic reasoning that its Pro-1 residue has the correct protonation state (pKₐ ~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 Pro-1 as a nucleophile to form an imine/enamine with various aldehyde and ketone compounds. The chemical potential of the smallest enamine, that generated from acetaldehyde, was then explored for further reactions 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 may 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. 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. 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.

\[ \text{CO}_2^- \xrightarrow{\text{CO}_2^-} \text{CO}_2^- \]

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

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) (Scheme 1). 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 \( \beta\alpha\beta \) structural fold. In the mechanism of 4-OT, Pro-1 is a general base that abstracts the 2-hydroxyl proton of 1 for delivery to the C-5 position, yielding 2. Pro-1 can function as a general base because the prolyl nitrogen has a \( pK_a \) of \( \sim 6.4 \) so that it exists largely as the uncharged species at pH 7.3. Two other key catalytic residues are Arg-11 and Arg-39. Arg-39 is proposed to interact with the 2-hydroxyl group of 1 and a C-1 carboxylate oxygen, whereas Arg-11 is proposed to interact with the C-6 carboxylate group in a bidentate fashion. The latter interaction may draw electron density toward C-5 for protonation by Pro-1.

Dawson and coworkers demonstrated that by mutating Pro-1 to an alanine, 4-OT can be changed from an acid/base tautomerase into an enzyme that decarboxylates oxaloacetate through a nucleophilic imine mechanism. This change in catalytic activity and mechanism is consistent with the expectation that the active site primary amine of Ala-1 would be less basic and more conformationally flexible than the secondary amine of Pro-1. This enables the nucleophilic addition of Ala-1 to oxaloacetate to form an iminium ion intermediate (i.e., a protonated Schiff base), which facilitates
decarboxylation. The decarboxylated product, pyruvate, is released from Ala-1 by hydrolysis.[8]

Different from this earlier work on mutant 4-OT, we set out to test whether the active site secondary amine of Pro-1 in wild-type 4-OT might be suitable for nucleophilic catalysis. We reasoned that although Pro-1 functions as a base catalyst in 4-OT’s natural activity, it has the correct protonation state ($pK_a \approx 6.4$) 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, Pro-1 may facilitate the reversible generation of enamines from carbonyl compounds (Scheme 2). Indeed, secondary amines react with carbonyl compounds to favor formation of enamine intermediates, while primary amines favor the imine tautomers.[10] The enzymatically generated enamine then could undergo a wide range of further reactions 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]

![Scheme 2](image)

**Scheme 2.** Proposed mechanism of enamine formation in the active site of 4-OT. Reduction of the imine intermediate by NaCNBH3 leads to irreversible covalent modification and inactivation of the enzyme.

**Results and discussion**

In order to identify reactive carbonyl compounds, possibly giving rise to useful enamines, 4-OT was incubated with various aldehydes and ketones (in separate reactions) and the mixtures were treated with NaCNBH3. 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 NaCNBH3 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, indicative of irreversible
covalent modification. Treatment of the enzyme with the aldehyde or NaCNBH₃ 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. While these compounds are reactive aldehydes with the potential of 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 pyruvyl moiety of 4-OT’s natural substrate 2.

**Scheme 3.** Carbonyl compounds tested as enamine donor (3-9) or as acceptor (10-13).

The inactivated protein samples were analyzed by ESI-MS in order 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 NaCNBH₃ (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 NaCNBH₃ (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), consistent with the proposed mechanism of enamine formation in the enzyme active site (Scheme 2).
Table 1. Inactivation and covalent modification of 4-OT by selected carbonyl compounds in the presence of NaCNBH₃.

<table>
<thead>
<tr>
<th>Enamine donor</th>
<th>Inactivation (%)</th>
<th>Calculated mass (Da)ᵃ</th>
<th>Observed mass (Da)</th>
<th>Covalent labeling (%)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>95</td>
<td>6839</td>
<td>6839</td>
<td>&gt;99</td>
</tr>
<tr>
<td>4</td>
<td>&gt;99</td>
<td>6853</td>
<td>6853</td>
<td>&gt;99</td>
</tr>
<tr>
<td>5</td>
<td>&gt;99</td>
<td>6867</td>
<td>6867</td>
<td>&gt;99</td>
</tr>
<tr>
<td>6</td>
<td>97</td>
<td>6881</td>
<td>6811ᵇ,c,6881</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>79</td>
<td>6867</td>
<td>6867</td>
<td>95</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>6854</td>
<td>6811ᵇ,c,6854</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>&gt;99</td>
<td>6883</td>
<td>6883</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

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.

We next investigated whether imine/enamine formation is an active site process that involves Pro-1. A 4-OT sample inactivated by 3 and a control sample (unmodified 4-OT) were digested with endoprotease 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 NaCNBH₃ to those of the unmodified 4-OT sample revealed a single modification by a species having a mass of 28 Da on the fragment Pro-1 to Glu-9 (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 Pro-1 as the sole site of labeling by 3 using Glu-C digestion and nano-LC-MS and MS/MS analyses.

<table>
<thead>
<tr>
<th>Peptide fragment</th>
<th>Calculated mass (Da)ᵃ</th>
<th>Observed mass (Da)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIAQIHILE</td>
<td>1033.6ᶜ</td>
<td>1032.5ᶜ</td>
</tr>
<tr>
<td>PIAQIH</td>
<td>660.4</td>
<td>660.4</td>
</tr>
<tr>
<td>PIAQ</td>
<td>410.2</td>
<td>410.2</td>
</tr>
<tr>
<td>PI</td>
<td>211.1</td>
<td>211.1</td>
</tr>
<tr>
<td>C₂H₅-PIAQIHILE</td>
<td>1061.6ᶜ</td>
<td>1060.6ᶜ</td>
</tr>
<tr>
<td>C₂H₅-PIAQIH</td>
<td>688.4</td>
<td>688.4</td>
</tr>
<tr>
<td>C₂H₅-PIAQ</td>
<td>438.2</td>
<td>438.2</td>
</tr>
<tr>
<td>C₂H₅-PI</td>
<td>239.1</td>
<td>239.1</td>
</tr>
</tbody>
</table>

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 Pro-1 to Glu-9, the most likely targets for alkylation are Pro-1 and His-6. 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$_2$H$_4$-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 Pro-1 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 Pro-1.

Encouraged by these findings, the chemical potential of the smallest enamine (that generated from 3) was explored for further reactions 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 S1A, Supporting Information). Integration of the signals indicates that ~10% of 3 and 10 had been converted to 15. While 15 has characteristic $^1$H NMR 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, Supporting Information). 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 μM or 180 μM of 4-OT, and the two reactions were followed by $^1$H NMR spectroscopy. Using 90 μM of 4-OT, ~4% and ~10% of 3 and 10 had been converted to 15 after 7 and 14 days, respectively. Using 180 μM of 4-OT, ~9% and ~16% of 3 and 10 had been 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 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, ruling out a nonenzymatic aldol condensation (Figure S1B, Supporting Information). 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, demonstrating 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 Pro-1,[13] led to single-site modification of 4-OT (as shown by ESI-MS) and the concomitant loss of the aldolase activity (Figure S3C, Supporting Information). 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, Supporting Information).

We next investigated the importance of Pro-1, Arg-11 and Arg-39 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, Supporting Information). These results provide further evidence indicating that 4-OT is responsible for the observed aldolase activity and that Pro-1 and Arg-11 are critical for the activity, whereas Arg-39 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 may 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 ($\lambda_{\text{max}} = 250$ nm), accompanied by the appearance of a new absorbance peak at 290 nm, which corresponds to 15 (Figure 1B). When the rate of this reaction is compared to that catalyzed by wild-type 4-OT (Figure 1A), 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, Supporting Information). Again, no product formation was detected for the control reaction without enzyme.

![Figure 1](image.png)

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

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, Supporting Information). Apparent $k_{\text{cat}}/K_m$ 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 ($k_{\text{cat}}/K_m = 1.4 \times 10^{-2}$ M$^{-1}$ s$^{-1}$) is 16-fold more efficient in catalyzing the aldol condensation of 3 with 10 than wild-type 4-OT ($k_{\text{cat}}/K_m = 8.5 \times 10^{-4}$ M$^{-1}$ s$^{-1}$). The improved aldolase activity of L8R-4-OT can also be clearly visualized using a phloroglucinol-based colorimetric assay,[16] which monitors the formation of 15 (Figure S5, Supporting Information).

In a possible mechanism that might explain the observed aldolase activity, Pro-1 functions as a nucleophile and attacks the carbonyl carbon of 3 to form an iminium ion (Scheme S1, Supporting Information). 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
could be stabilized by the active site arginine (i.e., Arg-11) known to be critical for the activity. This arginine may 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 may assist Arg-11 in charge stabilization, explaining the increased aldolase activity of this mutant enzyme.\textsuperscript{[17]}

Lineweaver-Burk plots at varying concentrations of 3 and different fixed values of 10 give a series of intersecting lines (Figure S4, Supporting Information). This pattern is characteristic of a sequential bi-substrate mechanism. As only substrate 3 can serve as enamine donor (10 does not have enolisable 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\textsubscript{3}, 4-OT is found to be labeled only partially by 10, whereas covalent modification by 3 is almost complete (Figure S6, Supporting Information). Taken together, the kinetic and labeling results are consistent with a sequential 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 \textsuperscript{1}H NMR spectroscopy demonstrate that this absorbance corresponds to the formation of 2-butenal (16, Scheme S2, Supporting Information). While 4-OT may 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 \textsuperscript{1}H NMR spectra monitoring the incubations of 3 and 10 with either 4-OT (Figure S1, Supporting Information) or the L8R-4-OT mutant (Figure S3F, Supporting Information). Additional experiments with synthetically prepared 4-OT and L8R-4-OT are underway to verify whether these enzymes also catalyze the self-condensation of 3 (rather than a contaminating protein).

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, which may 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,\textsuperscript{[18]} catalytic aldolase antibodies,\textsuperscript{[19]} and designed aldolase peptides.\textsuperscript{[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 may prove to be
a useful approach to discover new promiscuous carbonyl transformation activities that may 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 in order 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 Chemical Co. (St. Louis, MO), unless stated otherwise. The sources for the biochemicals, buffers, solvents, components of Luria-Bertani (LB) media 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 (Mannheim, Germany). 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, TX).

**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) 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 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$_2$O: δ = 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 using a Sciex API 3000 triple quadrupole mass spectrometer (AB Sciex, Concord, Ontario, Canada). The masses of peptide fragments in samples of Glu-C-digested 4-OT were determined by nano-LC-MS/MS 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)\(^{[15]} \) using the following primers:

- 5ʹ- A TAG CAG GTA **CAT** ATG CCT ATT GCC CAG ATC CAC ATC ATC CTT G-3ʹ (primer F, *NdeI* site underlined) and 5ʹ- G TGA TGT TAT GTA **TCC** TCA GCC TCT GAC CTT GCT GGC CAG TTC G-3ʹ (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 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: 5ʹ- A TAG CAG GTA **CAT** ATG GCC ATT GCC CAG ATC CAC ATC CTT G-3ʹ, 5ʹ- A TAG CAG GTA **CAT** ATG CCT ATT GCC CAG ATC CAC ATC **GCC** GAA GCC CGC AGC-3ʹ, and 5ʹ- A TAG CAG GTA **CAT** ATG CCT ATT GCC CAG ATC CAC ATC CTT GAA GCC **GCC** AGC GAC GAG CAG-3ʹ, respectively, where the *NdeI* site is underlined and the mutated codon is in bold. These primers correspond to the 5ʹ-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: 5ʹ- A TGT TAT GTA **TCC** TCA GCC TCT GAC CTT GCT GGC CAG TTC GCC GCC GAT GCC GAA GTG GCC CTT GGC CAT CTC CGT GAT AAT CAC **GCC** CAC GCT GG-3ʹ, where the *BamHI* site is underlined and the mutated codon is in bold. This primer corresponds to the 3ʹ-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) using the pET20b(+) expression system. LB medium (5 mL) containing ampicillin (Ap, 100 µg/ml) was inoculated with cells from a glycerol stock of *E. coli* BL21(DE3) containing the appropriate expression vector 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\(_2\)PO\(_4\) buffer (pH 8.0) (buffer A) and stored at -20 °C.

The 4-OT protein, either wild-type or mutant, was purified 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 as described above. 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 using Buffer A containing 0.5 M Na\(_2\)SO\(_4\) (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
(NH₄)₂SO₄. After stirring for 2 hr at 4 °C, the precipitate was removed by centrifugation
(20 min at 20,000 x g), and the supernatant was filtered and loaded onto a phenyl-
sepharose column (10 x 1.0 cm filled with ~8 mL of resin) that had been previously
equilibrated with Buffer A containing 1.6 M (NH₄)₂SO₄. The column was first washed
with the loading buffer (3 x 10 mL) and then the protein was eluted by gravity flow 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 NaH₂PO₄ buffer (pH 7.3) 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 (calc. 6810 Da). The observed monomer mass for the P1A mutant was 6786 Da
(calc. 6785 Da), that of the R11A mutant was 6727 Da (calc. 6726 Da), and that of the
R39A mutant was 6727 Da (calc. 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 (ε = 20 mM⁻¹ cm⁻¹) in 20 mM NaH₂PO₄ 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. An amount of 4-OT (2 mg) was incubated with
3-bromopyruvate (3-BP; final concentration 20 mM) in 20 mM NaH₂PO₄ buffer (pH 7.3)
for 1 h at 22 °C (total volume of 1 ml). Subsequently, NaBH₄ 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 NaH₂PO₄ buffer (pH 7.3). The proteins were eluted by gravity flow 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 (calc. 6900 Da). This 4-OT protein inactivated by 3-BP was used as a control in the $^1$H NMR assay for aldolase activity (see below).

**Sodium cyanoborohydride treatment of 4-OT in the presence of carbonyl compounds (3-10, 15).** An amount of 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$_2$PO$_4$ 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 water 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$_4$HCO$_3$ buffer (pH 8.0) using a pre-packed PD-10 Sephadex G-25 gel filtration 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.** An amount of 4-OT (0.5 mg) was incubated with 1 mM of acetaldehyde (3) in 20 mM NaH$_2$PO$_4$ 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 water 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$_2$PO$_4$ buffer (pH 8.0) 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, a quantity (50 μg) of unmodified 4-OT and 4-OT modified by 3 was vacuum-dried. The individual protein pellets from the two samples were dissolved in 10 μL of 10 M guanidine-HCl and incubated for 2 hrs at 37 °C. Subsequently, the samples were diluted by addition of 90 μL of 100 mM NH$_4$HCO$_3$ buffer (pH 8.0) and incubated for 2 days at 37 °C with protease GluC (2.5 μL from a 10 mg/mL stock solution in water). 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. The $^1$H NMR spectra monitoring the reactions between 3 and the four selected electrophiles were recorded as follows. A reaction mixture of 3 (50 mM) and either benzoaldehyde (10), acetophenone (11), cyclohexanecarboxyaldehyde (12) or cyclopentanecarboxyaldehyde (13) (each at ~50 mM) in 20 mM NaH$_2$PO$_4$ buffer (0.55 mL, pH 7.3) was placed in an NMR
tube, along with D$_2$O (0.05 mL) and 0.4 mg 4-OT (0.05 mL from a 8 mg/mL solution). Similar mixtures without 4-OT (the control samples) were prepared as well in order to analyze the non-enzymatic (uncatalyzed) reaction. $^1$H NMR spectra were recorded directly after mixing, and then after 1, 7 and 14 days.

The $^1$H NMR signals for 3, 10, 11, 12, and 13 are as follows. $^1$H NMR (500 MHz, 20 mM phosphate/D$_2$O buffer, pH 7.3) of 3 and its hydrate: $\delta$ = 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 (q, $J$ = 3.0 Hz, 1H). $^1$H NMR (500 MHz, 20 mM phosphate/D$_2$O buffer, pH 7.3) of 10: $\delta$ = 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 (s, 1H). $^1$H NMR (500 MHz, 20 mM phosphate/D$_2$O buffer, pH 7.3) of 11: $\delta$ = 2.52 (s, 3H), 7.42 (t, $J$ = 7.5 Hz, 2H), 7.55 (t, $J$ = 7.5 Hz, 1H), 7.85 (d, $J$ = 8.5 Hz, 2H). $^1$H NMR (500 MHz, 20 mM phosphate/D$_2$O buffer, pH 7.3) of 12: $\delta$ = 1.06-1.40 (m, 6H), 1.50-1.70 (m, 4H), 1.77 (m, 1H), 9.41 (s, 1H). $^1$H NMR (500 MHz, 20 mM phosphate/D$_2$O buffer, pH 7.3) of 13: $\delta$ = 1.20-1.54 (m, 6H), 1.58-1.76 (m, 2H), 2.27 (m, 1H), 9.45 (d, $J$ = 2.0 Hz, 1H).

In the incubation containing 3, 10, and 4-OT, extra signals were detected after 1 day, indicating the formation of cinnamaldehyde (15). The $^1$H NMR signals for 15 are as follows: $^1$H NMR (500 MHz, 20 mM phosphate/D$_2$O buffer, pH 7.3) of trans-15: $\delta$ = 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 (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$_2$O buffer, pH 7.3): $\delta$ = 1.91 (d, $J$ = 7.0 Hz, 1H), 6.07 (dd, $J$ = 8.0 Hz, 1H), 7.03 (m, 1H), 9.22 (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, Supporting Information].

Detection of 15 by UV spectroscopy and GC/MS analysis. While 15 has characteristic $^1$H NMR signals, its identity in the incubation mixture described above was further confirmed by UV spectroscopy and GC/MS analysis. Accordingly, after 14 days of incubation, a small aliquot was removed from the mixture containing 3, 10, and 4-OT and diluted 200-fold in 20 mM NaH$_2$PO$_4$ 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 ($\lambda_{\text{max}}$ = 250 nm, $\varepsilon_{250}$ = 15 mM$^{-1}$ cm$^{-1}$), the sample containing 4-OT showed extra peaks at $\lambda_{\text{max}}$ = 290 nm (15, $\varepsilon_{290}$ = 26.7 mM$^{-1}$ cm$^{-1}$) and around $\lambda_{\text{max}}$ = 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 (~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.
**1H NMR spectroscopy assay for aldolase activity.** 1H 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 NaH₂PO₄ buffer (pH 7.3) at 22 °C. In addition, to each tube 0.05 mL of D₂O 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 1H 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 1H 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, an amount (200 µg) of wild-type 4-OT, P1A-4-OT, R11A-4-OT, R39A-4-OT, L8R-4-OT, or synthetic 4-OT was incubated (in separate vials) with 3 and 10 (30 mM each) in 1.2 mL of 20 mM NaH₂PO₄ buffer (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 days, a sample of 50 µL was removed and mixed with 150 µL of 0.2% (w/v) phloroglucinol in 25/75 % (v/v) HCl/EtOH. 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 0.3 mL of 20 mM NaH₂PO₄ buffer (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) were plotted versus the concentration of 10 (mM) (Figure S4, Supporting Information). The slope of this plot is a straight line that equals (k_{cat} × [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 0.3 mL of 20 mM NaH₂PO₄ buffer (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) versus the reciprocal value of the concentration of 3 (mM⁻¹) (Figure S4, Supporting Information).
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Author contributions
E.Z. designed and performed most of the experiments, and analyzed the data. B.-J.B. developed the screening strategy. W.J.Q. and G.J.P. directed all aspects of the project. E.Z. and G.J.P. wrote the manuscript. All authors gave feedback on the manuscript.
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14. 4-OT was synthesized by GenScript USA Inc. (Piscataway, NY).
17. An alternative mechanism in which acetaldehyde will react via the derived enolate anion rather than via an enamine intermediate can not be excluded. In such a mechanism, the critical Pro-1 residue would function as a general base catalyst removing a proton from acetaldehyde to give the reactive enolate. However, this alternate mechanism seems unlikely in view of the ability of Pro-1 to rapidly react with acetaldehyde to form an iminium ion.


Supplementary information
Figures S1 – S7, schemes S1 and S2, and the corresponding legends, are provided below.

**Figure S1.** Partial $^1$H NMR spectra of the 4-OT-catalyzed (A) and uncatalyzed (B) aldol condensation of 3 with 10. Signals corresponding to the cross-condensation product cinnamaldehyde (15) are only observed in spectrum A, whereas signals corresponding to the self-condensation product 2-butenal (16) are observed in both spectra. The $^1$H NMR signals for 3, 10, 15 and 16 are reported in the experimental procedures.
Figure S2. GC-MS fragmentation pattern of the product formed in the incubation of 3 and 10 with 4-OT (top panel). The product was identified as cinnamaldehyde (15) based on the detection of fragment ions with masses highly similar to those found in MS/MS spectra of 15 in the NIST107 mass spectral reference library (two lower panels).
**Figure S3.** Partial $^1$H NMR spectra of the condensation of 3 with 10 catalyzed by P1A-4-OT (A), R11A-4-OT (B), 4-OT inactivated with 3-bromopyruvate (C), synthetic 4-OT (D), R39A-4-OT (E), and L8R-4-OT (F). Signals corresponding to the cross-condensation product cinnamaldehyde (15) are observed only in spectra D-F, whereas signals corresponding to the self-condensation product 2-butenal (16) are observed in all spectra. The $^1$H NMR signals for 3, 10, 15 and 16 are reported in the experimental procedures.

**Figure S4.** Kinetic analysis of the L8R-4-OT catalyzed condensation of 3 with 10. Left panel: a series of Lineweaver Burk plots of the reaction of 3 (AA) and 10 (BZ) catalyzed by L8R-4-OT (292 μM). The concentration of 10 is varying from 1 to 8 mM and the concentration of 3 is varying from 10 to 50 mM. Right panel: Michaelis-Menten plot of the reaction of 3 and 10 catalyzed by L8R-4-OT (145 μM). The initial rates were measured at a fixed concentration of 3 (50 mM) and varying concentrations of 10 (0.5 to 2 mM). 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). For wild-type 4-OT, the apparent $k_{cat}/K_m$ was determined in the same way.

**Figure S5.** Phloroglucinol-based colorimetric assay for the detection of cinnamaldehyde (15) in reaction mixtures of 3 and 10 incubated for 3 days with either a) an aliquot from a mock purification, b) 4-OT, c) L8R-4-OT, d) P1A-4-OT, e) R11A-4-OT, f) R39A-4-OT, g) synthetic 4-OT, and h) no enzyme added. A yellow color indicates the presence of 15.
Figure S6. ESI-MS spectra of 4-OT treated with A) 3 (1 mM; expected mass 6839 Da), B) 10 (1 mM; expected mass 6901 Da), and C) 15 (10 mM; expected mass 6930 Da). The mass of unlabeled 4-OT is 6810 Da. The deconvoluted spectra display a singly charged state.
Figure S7A. $^1$H NMR reference spectra of 3 and its hydrate (upper panel) and 10 (lower panel). The $^1$H NMR signals are reported in the experimental procedures.
Figure S7B. $^1$H NMR reference spectra of 15 (upper panel) and 16 (lower panel). The $^1$H NMR signals are reported in the experimental procedures.
Scheme S1. Proposed mechanism for the 4-OT-catalyzed condensation of 3 with 10 to yield 15.

Scheme S2. The self-condensation of acetaldehyde (3) to yield 2-butenal (16).