Biocatalytic Michael-type Additions of Acetaldehyde to Nitroolefins with the Proline-based Enzyme 4-Oxalocrotonate Tautomerase Yielding Enantioenriched γ-Nitroaldehydes

Yield up to 74% ee values up to 98%

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Abstract

Call me Michaelase

The enzyme 4-OT promiscuously catalyzes the Michael-type addition of acetaldehyde to a collection of aromatic and aliphatic nitroolefins with high stereoselectivity producing precursors of GABA analogues.
Introduction

γ-Nitroaldehydes are versatile and practical precursors for chiral γ-aminobutyric acids (GABAs). In particular, prominent GABA analogues such as marketed pharmaceuticals phenibut[1] (GABA\textsubscript{A} receptor agonist, anxiolytic), pregabalin[2] (anticonvulsant), baclofen[3] (GABA\textsubscript{B} receptor agonist, anti-alcoholism), and rolipram[4] (type IV phosphodiesterase inhibitor, antidepressant) can be readily obtained from diverse chiral γ-nitroaldehydes by two, well-precedented, chemical synthesis steps.[5] One of the most important strategies to construct γ-nitroaldehydes is the Michael-type addition of unmodified aldehydes to nitroolefins.[6] Following this approach, construction of the appropriate γ-nitroaldehyde precursors for above-mentioned, pharmaceutically active GABA analogues would require the Michael-type addition of acetaldehyde to various nitroolefin acceptors (Scheme 1). The Michael-type addition of unmodified aldehydes to nitroolefines has recently become viable by the development of proline- and peptide-based organocatalysts.[7,8] However, examples including acetaldehyde as donor are scarce since acetaldehyde is a relatively reactive and difficult to tame chemical and 10-20 mol% of organocatalyst is typically applied.[9] Alternative procedures for the asymmetric synthesis of γ-nitroaldehydes from acetaldehyde and nitroolefins are therefore of great interest. Although a few examples of enzyme-catalyzed carbon-carbon bond-forming Michael-type additions are known, these do not involve acetaldehyde as donor and mainly exhibit low stereoselectivities.[10]

Results and discussion

We here report that the enzyme 4-oxalocrotonate tautomerase (4-OT),[11] which carries a nucleophilic amino-terminal proline residue (Pro1), promiscuously catalyzes the asymmetric Michael-type addition of acetaldehyde to various aromatic and aliphatic nitroolefins yielding chiral γ-nitroaldehydes (Scheme 1) with high stereoselectivities. In combination with our previously described 4-OT-catalyzed addition of linear aldehydes

\[
\text{acetaldehyde} + \text{nitroolefin} \rightarrow \text{γ-nitroaldehyde}
\]

\[\begin{align*}
2a & \quad R = 3-\text{p-FC}-4-\text{MeC}_6\text{H}_3 & 3a & \quad (\text{precursor rolipram}) \\
2b & \quad R = 3-\text{OH}-4-\text{MeC}_6\text{H}_3 & 3b & \quad (\text{precursor rolipram}) \\
2c & \quad R = \text{iBu} & 3c & \quad (\text{precursor pregabalin}) \\
2d & \quad R = \text{PhCH=CH} & 3d & \quad \\
2e & \quad R = p-\text{ClC}_6\text{H}_4 & 3e & \quad (\text{precursor baclofen}) \\
2f & \quad R = p-\text{F-C}_6\text{H}_4 & 3f & \quad \\
2g & \quad R = \text{Ph} & 3g & \quad (\text{precursor phenibut})
\end{align*}\]

\[\text{2a-g}\]

Scheme 1. Michael-type addition of acetaldehyde 1 to nitroolefins 2a-g. * = chiral center.
Call me "miChaelase": exploiting electrophile scope

This is the first example of enzyme-catalyzed carbon-carbon bond-forming Michael-type additions that includes a range of linear aldehyde donors and a series of aromatic and aliphatic nitroolefin acceptors. Furthermore, we found that catalytic activity of 4-OT is preserved in aqueous solvent systems containing up to 50% (v/v) of DMSO as co-solvent. The 'Michaelase' activity of 4-OT and preservation of this activity in the presence of 50% (v/v) of an organic co-solvent are two important steps toward our aim of developing versatile and robust proline-based biocatalysts for carbon-carbon bond-forming Michael-type addition reactions.

The 4-OT-catalyzed Michael-type addition with donor acetaldehyde was explored with a series of nitroolefin acceptors (2a-f) in separate analytical scale experiments (Scheme 1). Nitroolefins 2a-f (0.7-3.0 mM) were incubated with acetaldehyde 1 (25-150 mM) and 4-OT (32-150 µM) in NaH₂PO₄ buffer (20 mM, pH 5.5) and a co-solvent. A co-solvent was required to achieve sufficient solubility of nitroolefins 2a-f in an aqueous solvent system. Apart from enhancing solubility of 2a-f, the co-solvent should be water-miscible, should not impede catalytic activity of 4-OT, and should not chemically react with any of the substrates (1 and 2a-f). Screening the activity of 4-OT in 20 mM NaH₂PO₄ buffer mixed with various amounts (5.0 to 72.5% v/v) of EtOH, DMSO, dioxane, THF, MeCN, and DMF revealed that EtOH (up to 10% v/v) and DMSO (up to 50% v/v) are suitable co-solvents that meet all above-mentioned criteria.

The analytical scale reactions were followed by monitoring the change of absorbance at λ_max of 2a-f by UV-spectroscopy. During all reactions, decrease of the absorbance at λ_max,2a-f was observed in course of time (20-120 min) indicating almost complete depletion of nitroolefins 2a-f (Figures S1-S6 in the Supporting Information). Identical experiments with 4-OT and 2a-f, respectively, in the absence of acetaldehyde 1 showed negligible decreases of absorbances at λ_max,2a-f (except for compound 2c, vide infra) demonstrating that acetaldehyde 1 is involved in the 4-OT-catalyzed conversions of 2a-f (Figures S1-S6 in the Supporting Information). These experiments also confirmed that EtOH and DMSO solely act as co-solvents and not as reagents. Three types of additional control experiments were executed to confirm that the enzyme 4-OT and its catalytic Pro1 residue are responsible for conversions of 2a-f and 1 (Figures S1-S6 in the Supporting Information). First, incubation of 1 with nitroolefins 2a-f, respectively, but in the absence of 4-OT did not result in any significant decreases of absorbances at λ_max,2a-f indicating that 4-OT is responsible for the catalytic activities. Second, experiments with 1 and 2a-f, respectively, in the presence of the P1A mutant of 4-OT showed no decreases of absorbances at λ_max,2a-f implying that the Pro1 residue is crucial for the catalytic activities of 4-OT. Third, 1 and 2a were incubated with synthetic 4-OT and the rate of decrease of absorbance at λ_max,2a was identical to that observed with recombinant 4-OT. Although highly purified recombinant 4-OT was used in the analytical assays, this finding eliminated the possibility that any contaminating proteins from the expression strain may be responsible for catalysis.

Preparative scale experiments were performed to allow unambiguous product identification by ¹H NMR spectroscopy and thus to ascertain that 4-OT-catalyzed conversions...
of 1 with 2a-f give Michael-type addition adducts 3a-f (Table 1). Nitroolefin (2a-f: 2-5 mM),[17] acetaldehyde (1, 50-150 mM),[16] and 4-OT (1.5-5.3 mol%)[16] were incubated in the appropriate solvent system (Table 1) and reactions were followed by UV spectroscopy. After disappearance of the absorbance at $\lambda_{max}$ of 2a-f, standard work-up and purification procedures were carried out which afforded γ-nitroaldehydes 3a-f as confirmed by $^1$H NMR spectroscopy. Yields between 49 and 74% were achieved for 3a-e while 3f was obtained in 26% yield. Products 3a-c,e are useful precursors for important GABA analogues since 3a-b can be converted into rolipram,[5c,20] 3c into pregabalin,[5b] and 3e into baclofen[5a,b,d-f] in two or three chemical synthesis steps, respectively. Furthermore, obtaining products 3a-f shows that 4-OT accepts aromatic as well as aliphatic nitroolefins as substrates for Michael-type addition reactions. The enantiomeric excesses (ee) of 3a-f were determined by GC or HPLC with chiral stationary phases. Excellent ee values between 95 and 98% were established for 3a, 3c, and 3d meaning that the enzyme 4-OT is highly stereoselective during the catalytic process. Obtained ee values of 3b,e,f range from 69 to 81%. The absolute configurations of the major enantiomers of 3a-f, respectively, were determined by HPLC and/or optical rotation.[17] Comparison with literature data revealed that the chiral centers of the major enantiomers of 3a-g, respectively, all have the identical geometry as depicted in Table 1. This means that the stereocontrol of 4-OT in the catalytic process of acetaldehyde addition to nitroolefins 3a-g is consistent regardless of the R-substituent (Scheme 1) at the nitroolefin. The major enantiomers of 3a,b,d-g have an (S)-configuration while 3c has an (R)-configuration. The deviant configuration of 3c is due to different prioritization of the substituents at the chiral center as compared to 3a,b,d-g. The amounts of applied 4-OT (1.5-3.7 mol%) were adjusted such that conversions of 2a,b,d-f were all completed within 2.5 h. Conversion of aliphatic substrate 2c was effected within 25 min due to the presence of 5.3 mol% of 4-OT. This amount of 4-OT was required to outcompete non-enzymatic water addition to 2c (giving racemic product 4-methyl-1-nitropentan-2-ol). Indeed, the amount of water addition product, 4-methyl-1-nitropentan-2-ol, went down from 4 to <2 mol% (compared to 3c) when 5.3 mol% of 4-OT was used instead of 2.6 mol% as determined by GC and $^1$H NMR spectroscopy. In contrast to 2c, non-enzymatic water addition to substrates 2a,b,d-f was not observed under the conditions used.

All preparative scale experiments of the 4-OT-catalyzed acetaldehyde addition to nitroolefins 2a-f were repeated under identical conditions but in the absence of 4-OT. In all cases no γ-nitroaldehyde product was observed (as confirmed by $^1$H NMR spectroscopy) demonstrating that formation of 3a-f is solely the result of 4-OT-catalyzed Michael-type additions and not of non-enzymatic addition of 1 to 2a-f. In case of 2c, non-enzymatic water addition resulted in formation of 4-methyl-1-nitropentan-2-ol as confirmed by $^1$H NMR spectroscopy and GC.
**Table 1.** Preparative scale 4-OT-catalyzed Michael-type addition reactions of acetaldehyde 1 (50-150 mM) to nitroolefins 2a-g (2-5 mM) in NaH₂PO₄ buffer (pH 5.5) yielding chiral γ-nitroaldehydes 3a-g.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Nitroolefin</th>
<th>Product (γ-nitroaldehyde)</th>
<th>t (h)</th>
<th>Yield[a] (%)</th>
<th>ee[b] (%)</th>
<th>Abs. Conf.[c]</th>
<th>4-OT (mol%)[d]</th>
<th>Co-solvent (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2a</td>
<td>3a</td>
<td>2.5</td>
<td>64</td>
<td>96</td>
<td>(S)</td>
<td>3.7</td>
<td>DMSO 40%</td>
</tr>
<tr>
<td>2</td>
<td>2b</td>
<td>3b</td>
<td>2.0</td>
<td>49</td>
<td>74</td>
<td>(S)</td>
<td>1.8</td>
<td>EtOH 10%</td>
</tr>
<tr>
<td>3</td>
<td>2c</td>
<td>3c</td>
<td>0.4</td>
<td>74</td>
<td>98</td>
<td>(R)</td>
<td>5.3</td>
<td>DMSO 5%</td>
</tr>
<tr>
<td>4</td>
<td>2d</td>
<td>3d</td>
<td>2.0</td>
<td>64</td>
<td>95</td>
<td>(S)</td>
<td>3.0</td>
<td>DMSO 40%</td>
</tr>
<tr>
<td>5</td>
<td>2e</td>
<td>3e</td>
<td>2.5</td>
<td>51</td>
<td>69</td>
<td>(S)</td>
<td>2.8</td>
<td>DMSO 45%</td>
</tr>
<tr>
<td>6</td>
<td>2f</td>
<td>3f</td>
<td>2.5</td>
<td>26</td>
<td>81</td>
<td>(S)</td>
<td>1.5</td>
<td>DMSO 40%</td>
</tr>
<tr>
<td>7[a]</td>
<td>2g</td>
<td>3g</td>
<td>2.0</td>
<td>70</td>
<td>81</td>
<td>(S)</td>
<td>1.4</td>
<td>EtOH 10%</td>
</tr>
</tbody>
</table>

[a] Isolated yields; [b] Determined by GC or HPLC with chiral stationary phase[c]; [c] Determined with HPLC with chiral stationary phase and/or optical rotation[d]; [d] Compared to nitroolefin; [e] Previous result
Conclusion

Summarizing, this work presents a biocatalytic methodology for asymmetric Michael-type additions of acetaldehyde to a collection of aliphatic and aromatic nitroolefin acceptors. The Michael-type additions are promiscuously catalyzed by the enzyme 4-OT and yield chiral γ-nitroaldehydes which are valuable precursors for GABA analogues. Yields up to 74% and ee values up to 98% were established demonstrating that 4-OT exerts high stereoselectivity during the catalytic process. Control experiments revealed that the ‘Michaelase’ activity takes place in the active site of 4-OT. The catalytic activity of 4-OT is preserved in aqueous solvent systems containing up to 50% DMSO (v/v). This finding implies that the substrate scope of our biocatalytic methodology is not limited to water-soluble chemicals and allows utilization of poorly water-soluble nitroolefins as substrates. The employed amounts of catalyst of 1.4–5.3 mol% in our methodology for Michael-type addition of acetaldehyde to nitroolefins are lower, and reactions times of ≤ 2.5 h are generally shorter, than in the scarce conventional organocatalytic methodologies for identical type of reactions.\[9,17\] Despite a relatively low molecular weight considering enzymes, the molecular mass of 4-OT is still considerably higher than those of organocatalysts\[9\] that are able to catalyze acetaldehyde addition to nitroolefins. Bearing this in mind, an alternative for defining catalyst efficiency on basis of the applied mol% of catalyst and reaction time is to assess catalyst efficiency by the weight amount of product (in terms of milligrams) that is produced per weight amount of used catalyst per unit of reaction time (mg\_product mg\_catalyst \textsuperscript{−1} h\textsuperscript{−1}). Applying the latter definition, 4-OT and the most potent organocatalyst\[9e\], to the best of our knowledge, are equally efficient in catalyzing the Michael-type addition of acetaldehyde (1) to nitrostyrene (2g).\[9,17,21\] This observation in combination with the broad substrate scope of our new enzyme-based methodology to prepare precursors of GABA analogues with high stereoselectivities inspired us to currently run protein engineering studies with the aim to enhance the unnatural ‘Michaelase’ activities of 4-OT. If successful, newly designed enzyme variants can also be tested in a whole cell system based on recombinantly expressed 4-OT, which appears to be an effective biocatalyst for the asymmetric Michael-type addition of acetaldehyde to a few selected aromatic β-nitrostyrenes.\[22\]

Acknowledgements

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6. Another elegant approach is the Michael-type addition of nitroalkanes to a,β-unsaturated carbonyls. See reference 5.


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*ChemBioChem* 2013, 14, 917 – 926.

J. Quax, G. J. Poelarends, *Angew. Chem.* 2012, 
124, 1266-1269; *Angew. Chem. Int. Ed.* 2012, 51, 
1240 – 1243.

13. Y. Miao, E. M. Geertsema, P. G. Tepper, E. 

14. The most likely catalytic mechanism of the 
4-OT-catalyzed Michael-type additions is 
reminiscent of proline-based organocatalysis 
and involves the formation of a nucleophilic 
enamine intermediate of the Pro1 residue of 
4-OT with acetaldehyde (Scheme S1). This 
intermediate reacts with the double bond of 
the nitroolefin acceptor creating a new carbon-
carbon bond after which the product is released 
from 4-OT’s Pro1 by hydrolysis.

15. The concentrations of 2a-f were adjusted on 
basis of their specific εₘₐₓ values and solubility 
properties. See SI for details.

16. Concentration of acetaldehyde and 4-OT 
adjusted on basis of concentration of nitroolefin 
(2a-f).

17. See SI for details.

18. 2a-f have different λₑₘ values. See SI for details.

19. Synthetic 4-OT was purchased from GenScript USA 
Inc. (Piscataway, NY). For folding of chemically 
synthesized 4-OT into active homohexamer see: 
M. C. Fitzgerald, I. Chernushевич, K. G. Standing, 

20. A Williamson ether synthesis of 3b with 
cyclopentanol gives 3a.

21. See reference 9e for the most efficient 
organocatalyst, to the best of our knowledge, for 
the Michael-type addition of acetaldehyde (1) to 
nitrostyrene (2g) in terms of the weight amount 
of product (3g in milligrams) that is produced 
per weight amount of applied catalyst per hour of 
reaction time (mg⁻¹ mg⁻¹ h⁻¹): (reference 9e) 
23.2 mg of organocatalyst catalyzes the Michael-
type addition of acetaldehyde (1) to nitrostyrene 
(2g) to give 54.2 mg of product 3g in 3 h of reaction 
time. This comes down to the production of 0.78 
mg of 3g per mg of catalyst per hour of reaction 
time (0.78 mg⁻¹ mg⁻¹ h⁻¹). In our methodology 
(Table 1 and reference 13) we use 11.3 mg of 4-OT 
for the Michael-type addition of acetaldehyde (1) 
to nitrostyrene (2g) to give 16.3 mg of product 3g 
in 2 h of reaction time. This comes down to the 
production of 0.72 mg of 3g per mg of catalyst 
per hour of reaction time (0.72 mg⁻¹ mg⁻¹ h⁻¹). 
See SI for a more detailed comparison of various 
aspects of our methodology with those presented 
in references 9a-f.

22. T. Narancic, J. Radivojevic, P. Jovanovic, D. 
Francuski, M. Bigovic, V. Maslak, V. Savic, B. 
Vasiljevic, K. E. O’Connor, J. Nikodinovic-Runic, 
Supporting information

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1. General information

Materials
All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise. The sources for the buffers, solvents, and components of Luria-Bertani (LB) media are reported elsewhere.[1] High purity synthetic 4-OT was obtained from GenScript USA Inc. (Piscataway, NY) and folded into the active homohexamer as described before.[2]

General methods
Techniques for transformation and other standard molecular biology manipulations were based on methods described elsewhere.[3] Proteins were 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 using the method of Waddell.[4] Enzymatic assays were monitored using a V-660 spectrophotometer purchased from Jasco (IJsselstein, The Netherlands). Evaporation of water/DMSO mixtures was performed with an acid-resistant CentriVap vacuum concentrator (Labconco, 78100 series) which was connected to a cold trap and a vacuum pump. NMR spectra were recorded on a Varian Inova 500 (500 MHz) or a Bruker DRX-500 (500 MHz) spectrometer. Chemical shifts for protons are reported in parts per million scale.
(δ scale) and are referenced to CHCl₃ (δ = 7.26). Reverse and normal phase HPLC was carried out using an in-house analytical HPLC equipped with a Shimadzu LC-10 AT pump and a Shimadzu SPD-M10A diode array detector using a Daicel Chiralpak AD-RH (reverse phase) or Chiralpak IB column (normal phase, Chiral Technologies Europe, Illkirch Cedex, France). The HPLC chromatographic data were analyzed by data processing software (LC Solutions) obtained from Shimadzu. Gas chromatography was carried out with a HP 5890 series II gas chromatograph (HP chiral 20% Permethylated beta-cyclodextrin column). Optical rotations were measured in CHCl₃ on a Schmidt + Haensch polarimeter (Polartronic MH8) with a 10 cm cell (c given in g/100 mL).

2. Expression and purification of proteins
The construction of the expression vectors and the purification procedure for wild type 4-OT and the P1A mutant were reported previously.[5] Wild type 4-OT enzyme and the P1A mutant were produced in E. coli BL21(DE3) using the pET20b(+) expression system as described before.[5]

3. Nitroolefins 2a-f
Nitroolefins 2e and 2f were commercially available while 2a,[6] 2b,[7] 2c,[8] and 2d[9] were synthesized and purified according to literature procedures.

4. Catalytic activity assay with 4-OT in water/co-solvent solvent systems
Nitroolefins 2a-f are insufficiently soluble in water to reach practical concentrations (≥ 0.5 mM) in 100% aqueous solvent systems for analytical and preparative scale 4-OT-catalyzed Michael-type addition reactions. Therefore, a co-solvent that enhances solubility of the nitroolefins to a practical level (≥ 0.5 mM) in aqueous solvent systems was required. Apart from enhancing solubility of 2a-f, the co-solvent should be water-miscible, should not impede catalytic activity of 4-OT, and should not chemically react with any of the substrates (1 and 2a-f). The following water-miscible solvents were tested for suitability: EtOH, DMSO, MeCN, THF, dioxane, and DMF. The experimental set up was as follows: the $k_{obs}$ of the 4-OT-catalyzed Michael-type addition of acetaldehyde 1 to t-nitrostyrene 2g in the presence of 5% co-solvent (i.e. 20 mM NaH₂PO₄ buffer/co-solvent 95/5 v/v) was determined by UV-spectroscopy (initial decay of absorbance at $\lambda_{max}$ of 2g (320 nm) in time). A minimum amount of co-solvent of 5% was required to get 2g into solution and to keep the Michael-type addition product 3g in solution after formation. Next, identical experiments were performed but with increased amounts of co-solvent of 10, 15, 30, 50, and 72.5% respectively. The concentrations of 4-OT (32.5 µM, 2.5 mol% compared to 2g), 1 (65 mM), and 2g (1.3 mM) and final volumes (0.3 mL) were identical in all experiments. This means that catalytic activities of 4-OT in the presence of different co-solvents could be compared with each other and that the influence of increasing volume percentages of the different co-solvents on the activities of 4-OT could be determined.
The results are summarized in Table S1 and show that the presence of 5% (v/v) of co-solvents EtOH, DMSO, MeCN, THF, dioxane, and DMF resulted in comparable values of $k_{\text{obs}}$ between $3.5 \times 10^{-2}$ s$^{-1}$ and $5.1 \times 10^{-2}$ s$^{-1}$ for the 4-OT-catalyzed Michael-type addition of 1 to 2g in 20 mM NaH$_2$PO$_4$ buffer (pH 5.5). An increase of the co-solvents to 10% (v/v) did not result in significant changes in any of the $k_{\text{obs}}$ values (3.6-5.1 $\times 10^{-2}$ s$^{-1}$). Increasing the amount of co-solvent to 15% (v/v) significantly reduced the catalytic activity of 4-OT in dioxane, THF, and MeCN while catalytic activity in EtOH, DMSO, and DMF was more or less preserved. Increasing the percentage of co-solvent to 30% (v/v) reduced activity of 4-OT in dioxane, THF, and MeCN to practically zero while activities were roughly halved in EtOH and DMF. In 30% (v/v) DMSO, activity was retained. Increase of co-solvent to 50% (v/v) showed a further decrease of catalytic activity in EtOH and DMF while activity in 50% (v/v) DMSO remained at the identical level as in 5% (v/v) DMSO. When the percentage of DMSO was increased to 72.5% (v/v), significant decrease in catalytic activity was observed. Based on these results, DMSO was nominated as a suitable co-solvent for the 4-OT-catalyzed Michael-type addition reactions of acetaldehyde and nitroolefins 2a-f. Summarizing, DMSO was nominated since it is water-miscible, increases the solubility of 2a-f (vide infra) in aqueous solvent systems and does not impede the catalytic activity of 4-OT in aqueous solvent systems when present up to 50% (v/v).

**Table S1.** $k_{\text{obs}}$ for 4-OT-catalyzed Michael-type addition of 1 to 2g in 20 mM NaH$_2$PO$_4$ (pH 5.5) in the presence of various amounts (v/v) of co-solvents EtOH, DMSO, DMF, dioxane, THF, and MeCN. Identical initial concentrations of 1 (c = 65 mM), 2g (c = 1.3 mM), and 4-OT (c = 32.5 µM) were applied in all experiments. $k_{\text{obs}} = D[2g]_{initial}/[4-OT]$

<table>
<thead>
<tr>
<th>solvent</th>
<th>5% (v/v)</th>
<th>10% (v/v)</th>
<th>15% (v/v)</th>
<th>30% (v/v)</th>
<th>50% (v/v)</th>
<th>72.5% (v/v)</th>
</tr>
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<tbody>
<tr>
<td>EtOH</td>
<td>5.1</td>
<td>5.1</td>
<td>4.4</td>
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<td>3.9</td>
<td>3.2</td>
<td>2.2</td>
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<td>0.6</td>
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<td>3.7</td>
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</tr>
<tr>
<td>THF</td>
<td>3.5</td>
<td>3.6</td>
<td>1.7</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>MeCN</td>
<td>4.0</td>
<td>4.2</td>
<td>1.9</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
</tr>
</tbody>
</table>

The solubility of nitroolefins 2a-f was tested in several water/co-solvent solvent systems which revealed that nitroolefin 2b is soluble to at least 2.0 mM in a water/10% EtOH (v/v) solvent system while nitroolefins 2a,c-f require between 5 and 45% (v/v) presence...
of co-solvent DMSO to be soluble to at least 2.0 mM in aqueous solvent systems. The applied percentages of EtOH and DMSO as co-solvents in analytical and preparative scale experiments are listed in Tables 1 (main text), S2, and S3.

Analytical scale control experiments ascertained that EtOH and DMSO solely act as co-solvents in the 4-OT-catalyzed depletion of nitroolefins 2a-f and not as reagents (see main text for explanation and Section 5, Figures S1-S6).

5. Analytical scale 4-OT-catalyzed Michael-type addition reactions

*General procedure for analytical scale reactions*

The UV-spectroscopic assays to monitor the 4-OT-catalyzed Michael-type addition reactions were performed at 22°C by following the decrease in absorbance at λ<sub>max</sub> of the nitroolefin (2a-f) in course of time which corresponds to the depletion of the nitroolefin (see Table S2 for specific λ<sub>max</sub> values of 2a-f).

A stock solution of acetaldehyde (1) was prepared in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 5.5), while separate stock solutions of nitroolefins 2a-f were prepared in absolute ethanol or DMSO (see Table S2). The rationale behind applying ethanol and DMSO as co-solvents is explained in detail in Section 4. An aliquot of enzyme (4-OT) and acetaldehyde (1) were incubated in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 5.5) in a 1 mm cuvette, after which the assay was initiated by the addition of the nitroolefin (2a-f). The resulting final concentrations of nitroolefin, acetaldehyde, and enzyme were as indicated in Table S2. The final concentrations of nitroolefins were generally adjusted on basis of their specific ε<sub>max</sub> values (i.e. the lower the ε<sub>max</sub> value, the higher the final concentration: 1.9 < Absorbance at λ<sub>max</sub> < 2.9 except for 2a). The total volume of the reaction mixtures was 0.3 mL and the final ratios of water/EtOH or water/DMSO were as indicated in Table S2 for each specific nitroolefin. The reactions were monitored in course of time by recording absorbance spectra from 200 to 400, or 200-600 nm, and the spectra were analyzed using the software provided with the UV-VIS spectrophotometer (Jasco). The following control experiments were run for each nitroolefin; 1) conditions as in Table S2 but in the absence of 4-OT; 2) conditions as in Table S2 but in the absence of acetaldehyde 1; 3)

**Table S2. Conditions of analytical scale, 4-OT-catalyzed Michael-type additions of acetaldehyde 1 to nitroolefins 2a-f in 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 5.5): λ<sub>max</sub> and ε<sub>max</sub> of nitroolefins 2a-2f.**

<table>
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<tr>
<th>entry</th>
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<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>ε&lt;sub&gt;max&lt;/sub&gt; (mM&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>solvent stock solution</th>
<th>final conc. nitroolefin (mM)</th>
<th>final conc. 1 (mM)</th>
<th>final conc. 4-OT (µM)</th>
<th>co-solvent (v/v)</th>
<th>t&lt;sub&gt;final&lt;/sub&gt; (h)</th>
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conditions as in Table S2 but in the presence of 4-OT P1A instead of 4-OT wild-type. During all these control experiments no significant decreases of absorbances at \( \lambda_{\text{max}} \) values of 2a-f (except for 2c, see main text) were observed (see Figures S1-S6).

A control experiment with synthetic 4-OT was performed for the reaction of acetaldehyde (1) with nitroolefin 2a. Fully folded synthetic 4-OT was prepared as described elsewhere.[2] The folded synthetic 4-OT was incubated with 1 and 2a under the same conditions as those for the experiment with recombinant 4-OT (Table S2, entry 1). The reaction was monitored for the same period of time as for the experiment with recombinant 4-OT and a similar decrease of absorbance at 378 nm, corresponding to the disappearance of 2a, was observed (Figure S1).

UV-spectra, recorded after appropriate time intervals, to monitor the progress of analytical scale experiments and UV time course measurements of the control experiments, monitoring the change in absorbance at \( \lambda_{\text{max}} \) of the respective nitroolefin, are visualized in Figures S1-S6.

Figure S1. UV-spectra (left) showing the depletion of nitroolefin 2a incubated with 1 and recombinant 4-OT in 20 mM NaH\(_2\)PO\(_4\) buffer/40% DMSO (v/v) at pH 5.5; UV-spectra (right) following the depletion of 2a at 378 nm in the presence of 1 and 4-OT P1A (□), recombinant 4-OT (▲), synthetic 4-OT (◊), in the presence of 4-OT but the absence of 1 (●), and without 4-OT (Blank, ○).
Figure S2. UV-spectra (left) showing the depletion of nitroolefin 2b incubated with 1 and recombinant 4-OT in 20 mM NaH₂PO₄ buffer/10% EtOH (v/v) at pH 5.5; UV-spectra (right) following the depletion of 2b at 375 nm in the presence of 1 and 4-OT P1A (□), recombinant 4-OT (▲), in the presence of 4-OT but the absence of 1 (●), and without 4-OT (Blank, ○).

Figure S3. UV-spectra (left) showing the depletion of nitroolefin 2c incubated with 1 and recombinant 4-OT in 20 mM NaH₂PO₄ buffer/5% DMSO (v/v) at pH 5.5; UV-spectra (right) following the depletion of 2c at 249 nm in the presence of 1 and 4-OT P1A (□), recombinant 4-OT (▲), in the presence of 4-OT but the absence of 1 (●), and without 4-OT (Blank, ○).
**Figure S4.** UV-spectra (left) showing the depletion of nitroolefin 2d incubated with 1 and recombinant 4-OT in 20 mM NaH₂PO₄ buffer/40% DMSO (v/v) at pH 5.5; UV-spectra (right) following the depletion of 2d at 362 nm in the presence of 1 and 4-OT P1A (□), recombinant 4-OT (▲), in the presence of 4-OT but the absence of 1 (●), and without 4-OT (Blank, ○).

**Figure S5.** UV-spectra (left) showing the depletion of nitroolefin 2e incubated with 1 and recombinant 4-OT in 20 mM NaH₂PO₄ buffer/45% DMSO (v/v) at pH 5.5; UV-spectra (right) following the depletion of 2e at 320 nm in the presence of 1 and 4-OT P1A (□), recombinant 4-OT (▲), in the presence of 4-OT but the absence of 1 (●), and without 4-OT (Blank, ○).
6. Preparative scale reactions, characterization, and enantiomeric excess determination of 3a-f

The conditions of preparative scale, 4-OT-catalyzed Michael-type additions of acetaldehyde 1 to nitroolefins 2a-f are summarized in Table S3. Specific details can be found in the procedures following Table S3. The rationale behind applying ethanol and DMSO as co-solvents is explained in detail in Section 4. After work-up procedures, products 3a,c-f were characterized by recording 1H NMR spectra and comparison with data in the literature (see specific procedures following Table S3 for references). Product 3b has been characterized by 1H NMR, 13C NMR, and exact mass spectroscopy since, to the best of our knowledge, 3b has not been described in the literature. The enantiomeric excess of 3c was determined by GC with a chiral stationary phase (HP chiral 20% Permethylated beta-cyclodextrin column). Enantiomeric excesses of products 3a,b,d-f were determined by reverse or normal phase HPLC with a chiral stationary phase. The aldehyde functionality of 3a was derivatized into a methyl ester according to a literature procedure to be able to determine the enantiomeric excess.10 The aldehyde functionality of 3b was derivatized into a cyclic acetal to be able to determine the enantiomeric excess (see Section 8 for details). As control experiments, blank reactions of acetaldehyde and nitroolefin without enzyme were performed under the exact same conditions as described in Table S3. No formation of products 3a-f was observed in any of these control experiments. During the control experiment with 2c, formation of side product 4-methyl-1-nitropentane-2-ol was observed as a result of non-enzymatic water addition to 2c. Analysis of this side product, and of chemically obtained 4-methyl-1-nitropentane-2-ol, with GC with a chiral stationary phase indeed revealed that side product 4-methyl-1-nitropentane-2-ol was obtained racemic (HP chiral 20% Permethylated beta-cyclodextrin column, 95°C isocratic, 1 mL min⁻¹, tR: = 99.8 and 112.8 min.)
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Table S3. Conditions of preparative scale, 4-OT-catalyzed Michael-type additions of acetaldehyde 1 to nitroolefins 2a-f in 20 mM NaH₂PO₄ buffer (pH 5.5)

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<th>final conc. 1 (mM)</th>
<th>4-OT (µM)</th>
<th>4-OT (mol%)</th>
<th>co-solvent (v/v)</th>
<th>t_{final} (h)</th>
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3-(3-(cyclopentylxylo)-4-methoxyphenyl)-4-nitrobutanal (3a)

A solution of nitroolefin 2a (31.6 mg, 0.12 mmol) in DMSO (6.0 mL) was added to a mixture of acetaldehyde 1 (132 mg, 3.0 mmol) and 4-OT (30 mg, 4.4×10⁻³ mmol, 3.7 mol%) in 20 mM NaH₂PO₄ buffer pH 5.5 and DMSO (final volume mixture: 60.0 mL. Final ratio water/DMSO: 60/40 v/v). The mixture was incubated at 22°C and reaction progress was monitored by recording UV-spectra of aliquots taken from the reaction mixture after regular time intervals. After 2.5 h, all 2a was converted and the reaction mixture was divided over six polypropylene tubes (15 mL, CELLSTAR). The solvents were evaporated using an acid-resistant CentriVap vacuum concentrator (55°C, overnight). Water (3 mL) and chloroform (3 mL) were added to each tube. The dry residues were dissolved/suspended by vigorous stirring (additional scraping with a spatula may be required). The combined water and chloroform layers were separated in a separatory funnel. The water layer was extracted with chloroform (3×10 mL). The combined organic layers were dried with MgSO₄ and concentrated in vacuo to yield 3a (23.6 mg, 7.7×10⁻² mmol, 64%) as a colorless oil. The ¹H NMR spectroscopic data of 3a are in agreement with published data.¹¹ Enantiomeric excess was determined by normal phase HPLC with derivatized 3a using a Chiralpak IB column (n-heptane/i-PrOH 95:5, 25°C) at 1 mL/min, UV detection at 220 nm: t_{R}: (minor) = 32.2 min, (major) = 34.9 min.
3-(3-hydroxy-4-methoxyphenyl)-4-nitrobutanal (3b)

A solution of nitroolefin 2b (23.4 mg, 0.12 mmol) in ethanol (6.0 mL) was added to a mixture of acetaldehyde 1 (132 mg, 3.0 mmol) and 4-OT (15 mg, 2.2×10⁻³ mmol, 1.8 mol%) in 20 mM NaH₂PO₄ buffer pH 5.5 (final volume mixture: 60.0 mL). The reaction mixture was incubated at 22°C and reaction progress was monitored by recording UV-spectra of aliquots taken from the reaction mixture after regular time intervals. After 2 h, all 2b was converted and the reaction mixture was centrifuged using a Vivaspin column (Sartorius Stedim Biotech S.A., France) with a 5000 Da molecular weight cut-off filter to remove 4-OT. The flow-through was collected and extracted with ethyl acetate (3×25 mL). The combined organic layers were dried with brine (25 mL) and MgSO₄ and concentrated in vacuo to yield 3b (14.1 mg, 5.9×10⁻² mmol, 49%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃, 25°C) δ 9.69 (t, J = 1.2 Hz, 1H), 6.80 (d, J = 8.2 Hz, 1H), 6.78 (d, J = 2.3 Hz, 1H), 6.72 (dd, J = 8.2, 2.3 Hz, 1H), 5.63 (b, 1H), 4.62 (dd, J = 12.4, 7.2 Hz, 1H), 4.56 (dd, J = 12.4, 7.6 Hz, 1H), 3.98 (dddd, J = 7.6, 7.6, 6.8 Hz, 1H), 3.87 (s, 3H), 2.91 (ddd, J = 18.0, 7.6, 1.2 Hz, 1H), 2.87 (ddd, J = 18.0, 6.8, 1.2 Hz, 1H); ¹³C NMR (125 MHz, CDCl₃, 25°C) δ 199.02, 146.26, 146.06, 131.09, 119.26, 113.14, 111.00, 79.58, 55.91, 46.38, 37.54; HRMS (ESI): m/z = 240.08655 [M+H]+ (calcd. 240.08665 for C₁₁H₁₄NO₅); Enantiomeric excess was determined by reverse phase HPLC with derivatized 3b using a Chiracel AD-RH column (MeCN/water 40:60, 25°C) at 0.8 mL/min, UV detection at 220 nm: tᵣ: (minor) = 19.9 min, (major) = 29.3 min.

5-methyl-3-(nitromethyl)hexanal (3c)

A solution of nitroolefin 2c (5.0 mg, 3.9×10⁻² mmol) in DMSO (0.64 mL) was added to a mixture of acetaldehyde 1 (85.3 mg, 1.94 mmol) and 4-OT (14.1 mg, 2.07×10⁻³ mmol, 5.3 mol%) in 20 mM NaH₂PO₄ buffer pH 5.5 (final volume mixture: 12.8 mL). The mixture was left at 22°C and after 25 min the mixture was extracted with toluene (3×10 mL). The combined organic layers were dried (Na₂SO₄) and concentrated in vacuo to yield 3c (5.0 mg, 2.9×10⁻² mmol, 74%) as a colorless oil. The ¹H NMR spectroscopic data of 3c are in agreement with published data. Enantiomeric excess was determined by GC (HP chiral 20% Permethylated beta-cyclodextrin column, 95°C isocratic, 1 mL min⁻¹, tᵣ: (minor) = 110.2 min, (major) = 112.8 min.)
A solution of nitroolefin 2d (21.9 mg, 1.25×10⁻¹ mmol) in DMSO (10 mL) was added to a mixture of acetaldehyde 1 (55.1 mg, 1.25 mmol) and 4-OT (25.5 mg, 3.74×10⁻³ mmol, 3.0 mol%) in 20 mM NaH₂PO₄ buffer pH 5.5 (final volume mixture: 25.0 mL. Final ratio water/DMSO: 60/40 v/v). The mixture was left at 22°C and reaction progress was monitored by recording UV-spectra of aliquots taken from the reaction mixture after regular time intervals. After 2.0 h, all 2d was converted and the reaction mixture was divided over six polypropylene tubes (15 mL, CELLSTAR). The solvents were evaporated using an acid-resistant CentriVap vacuum concentrator (55°C, overnight). Water (3 mL) and chloroform (3 mL) were added to each tube. The dry residues were dissolved/suspended by vigorous stirring (additional scraping with a spatula may be required). The combined water and chloroform layers were separated in a separatory funnel. The water layer was extracted with chloroform (3×10 mL). The combined organic layers were dried with MgSO₄ and concentrated in vacuo to yield 3d (17.6 mg, 8.0×10⁻² mmol, 64%), and slight amounts of impurities (~10% compared to 3d), as a yellowish oil. The ¹H NMR spectroscopic data of 3d are in agreement with published data.[13] Enantiomeric excess was determined by reverse phase HPLC using a Chiracel AD-RH column (MeCN/water 40:60, 25°C) at 0.8 mL/min, UV detection at 210 nm: tᵣ: (minor) = 6.9 min, (major) = 7.4 min.

3-(4-chlorophenyl)-4-nitrobutanal (3e)

A solution of nitroolefin 2e (12.0 mg, 6.5×10⁻² mmol) in DMSO (2.5 mL) was added to a mixture of 4-OT (12.5 mg, 1.8×10⁻³ mmol, 2.8 mol%) in 20 mM NaH₂PO₄ buffer pH 5.5 and DMSO. Acetaldehyde 1 (143 mg, 3.25 mmol) was added and the mixture was left at 22°C (final volume mixture: 50.0 mL. Final ratio water/DMSO: 55/45 v/v). Reaction progress was monitored by recording UV-spectra of aliquots taken from the reaction mixture after regular time intervals. After 2.5 h, all 2e was converted and the reaction mixture was divided over six polypropylene tubes (15 mL, CELLSTAR). The solvents were evaporated using an acid-resistant CentriVap vacuum concentrator (55°C, overnight). Water (3 mL) and chloroform (3 mL) were added to each tube. The dry residues were dissolved/suspended by vigorous stirring (additional scraping with a spatula may be required). The combined water and chloroform
layers were separated in a separatory funnel. The water layer was extracted with chloroform (3×10 mL). The combined organic layers were dried with MgSO₄ and concentrated *in vacuo* to yield 3e (7.5 mg, 3.3×10⁻² mmol, 51%) as a colorless oil. The ¹H NMR spectroscopic data of 3e are in agreement with published data.[¹²,¹⁴] Enantiomeric excess was determined by reverse phase HPLC using a Chiracel AD-RH column (MeCN/water 32:68, 22°C) at 0.5 mL/min, UV detection at 220 nm: \( t\_R \) (minor) = 37.3 min, (major) = 40.0 min.

3-(4-fluorophenyl)-4-nitrobutanal (3f)

A solution of nitroolefin 2f (20.1 mg, 0.12 mmol) in DMSO (6.0 mL) was added to a mixture of acetaldehyde 1 (132 mg, 3.0 mmol) and 4-OT (12.0 mg, 1.8×10⁻³ mmol, 1.5 mol%) in 20 mM NaH₂PO₄ buffer pH 5.5 and DMSO. The mixture was left at 22°C (final volume mixture: 60.0 mL. Final ratio water/DMSO: 60/40 v/v). Reaction progress was monitored by recording UV-spectra of aliquots taken from the reaction mixture after regular time intervals. After 2.5 h, all 2f was converted and the reaction mixture was divided over six polypropylene tubes (15 mL, CELLSTAR). The solvents were evaporated using an acid-resistant CentriVap vacuum concentrator (55°C, overnight). Water (3 mL) and chloroform (3 mL) were added to each tube. The dry residues were dissolved/suspended by vigorous stirring (additional scraping with a spatula may be required). The combined water and chloroform layers were separated in a separatory funnel. The water layer was extracted with chloroform (3×10 mL). The combined organic layers were dried with Na₂SO₄ and concentrated *in vacuo* to yield 3f (6.7 mg, 3.2×10⁻² mmol, 26%) as a colorless oil. The ¹H NMR spectroscopic data of 3f are in agreement with published data.[¹⁴] Enantiomeric excess was determined by reverse phase HPLC using a Chiracel AD-RH column (MeCN/water 30:70, 25°C) at 0.5 mL/min, UV detection at 210 nm: \( t\_R \) (minor) = 34.1 min, (major) = 35.9 min.

7. Synthesis of racemic 3a-f for enantiomeric excess determinations

General procedure: racemic 3a-f were synthesized to serve as references for enantiomeric excess determinations of enzymatically obtained 3a-f. Racemic 3a-f were synthesized with the following general procedure: under a nitrogen atmosphere, nitroolefin, piperidine (0.2 eq) and acetaldehyde (10 eq) were dissolved in THF or MeOH (3 mL). The solution was stirred at room temperature for 48 h. The reaction progress was monitored by thin layer chromatography (TLC, silica, n-heptane/EtOAc, visualization: KMnO₄). The solvent was evaporated and the residue purified by column chromatography (silica gel, n-heptane/AcOEt). The ¹H NMR spectroscopic data of 3a-c-f were in agreement with published data: 3a,[¹¹] 3c,[¹²] 3d,[¹³] 3e,[¹²,¹⁴] 3f.[¹⁴]
3-(3-(cyclopentyloxy)-4-methoxyphenyl)-4-nitrobutanal (3a) Nitroolefin 2a (213 mg, 0.81 mmol) gave 3a (17 mg, 5.5×10⁻² mmol, 7%) after column chromatography (silica gel, n-heptane/AcOEt 4/1). Reaction solvent: MeOH.

3-(3-hydroxy-4-methoxyphenyl)-4-nitrobutanal (3b) Nitroolefin 2b (158 mg, 0.81 mmol) gave 3b (28 mg, 0.12 mmol, 14%) after column chromatography (silica gel, n-heptane/AcOEt 2/1). Reaction solvent: MeOH. ¹H NMR spectroscopic data are in agreement with the data from enzymatically obtained 3b.

5-methyl-3-(nitromethyl)hexanal (3c) Nitroolefin 2c (100 mg, 0.77 mmol) gave 3c (20 mg, 0.12 mmol, 15%) after column chromatography (silica gel, n-heptane/AcOEt 4/1). Reaction solvent: THF.

(E)-3-(nitromethyl)-5-phenylpent-4-enal (3d) Note: reaction time was 240 h. Nitroolefin 2d (100 mg, 0.57 mmol) gave 3d (2.4 mg, 1.1×10⁻² mmol, 2%) after column chromatography (silica gel, n-heptane/AcOEt 95/5 → 50/50). Reaction solvent: MeOH.

3-(4-chlorophenyl)-4-nitrobutanal (3e) Nitroolefin 2e (183 mg, 1.00 mmol) gave 3e (41 mg, 0.18 mmol, 18%) after column chromatography (silica gel, n-heptane/AcOEt 3/1). Reaction solvent: MeOH.

3-(4-fluorophenyl)-4-nitrobutanal (3f) Nitroolefin 2f (135 mg, 0.81 mmol) gave 3f (30 mg, 0.14 mmol, 18%) after column chromatography (silica gel, n-heptane/AcOEt 4/1). Reaction solvent: MeOH.

8. Derivatization of 3a and 3b for enantiomeric excess determinations

Methyl 3-(3-(cyclopentyloxy)-4-methoxyphenyl)-4-nitrobutanoate (4a)

The aldehyde functionalities of enzymatically obtained 3a and racemic 3a were converted into methyl esters according to a literature procedure.[10]
5-(1-(1,3-dioxolan-2-yl)-3-nitropropan-2-yl)-2-methoxyphenol (4b)

Under a nitrogen atmosphere, enzymatically obtained 3b (12.5 mg, 5.2×10^{-2} mmol), ethylene glycol (64 mg, 1.0 mmol), and p-TsOH (5 mol%, 0.5 mg, 2.6×10^{-3} mmol) were stirred in chloroform (2 mL) for 4 d at room temperature. Reaction progress was monitored by thin layer chromatography (silica, hexanes/EtOAc 1/1, R_f product = 0.31, R_f starting material = 0.25; visualization KMnO_4). The reaction mixture was concentrated and purified by column chromatography (silica gel, hexanes/EtOAc 2/1) to give 4b (5.0 mg, 1.8×10^{-2} mmol, 34%) as a colorless oil. 1H NMR (300 MHz, CDCl_3, 25°C) δ 6.79 (d, J = 2.2 Hz, 1H), 6.78 (d, J = 8.4 Hz, 1H), 6.70 (dd, J = 8.4, 2.2 Hz, 1H), 5.61 (b, 1H), 4.74 (dd, J = 6.5, 3.1 Hz, 1H), 4.70 (dd, J = 12.5, 6.5 Hz, 1H), 4.70 (dd, J = 12.5, 9.0 Hz, 1H), 3.99-3.93 (m, 2H), 3.87 (s, 3H), 3.84-3.77 (m, 2H), 3.66 (dddd, J = 9.0, 8.0, 6.5, 6.5 Hz, 1H), 2.07 (ddd, J = 14.0, 8.0, 3.1 Hz, 1H), 1.92 (ddd, J = 14.0, 6.5, 6.5 Hz, 1H); HRMS (ESI): m/z = 284.11249 [M+H]^+ (calcd. 284.11286 for C_{13}H_{18}NO_6)

Identical procedure was carried out with racemic 3b.
9. HPLC and GC chromatograms of enantiomeric excess determinations

**Figure S7.** HPLC chromatograms of racemic 4a and enzymatically obtained 4a.

**Figure S8.** HPLC chromatograms of racemic 4b and enzymatically obtained 4b.
Figure S9. GC chromatograms of racemic 3c and enzymatically obtained 3c.

Figure S10. HPLC chromatograms of racemic 3d and enzymatically obtained 3d.
Figure S11. HPLC chromatograms of racemic 3e and enzymatically obtained 3e.

Figure S12. HPLC chromatograms of racemic 3f and enzymatically obtained 3f.
10. $^1$H NMR spectra of enzymatically obtained 3a-f and derivatized 3b

**Figure S13.** $^1$H NMR spectrum of enzymatically obtained 3a.

**Figure S14.** $^1$H NMR spectrum of enzymatically obtained 3b.
Figure S15. $^1$H NMR spectrum of enzymatically obtained 3c.

Figure S16. $^1$H NMR spectrum of enzymatically obtained 3d.
Figure S17. $^1$H NMR spectrum of enzymatically obtained 3e.

Figure S18. $^1$H NMR spectrum of enzymatically obtained 3f.
Figure S19. $^1$H NMR spectrum of 4b, the cyclic acetal synthesized from enzymatically obtained 3b.
11. Proposed reaction mechanism of 4-OT-catalyzed Michael-type addition of acetaldehyde 1 to nitroolefins 2a-g

\[
\begin{align*}
\text{acetaldehyde 1} & \quad \overset{\text{Pro-1}}{\begin{array}{c}
\text{4-OT} \\
\text{O} \\
\text{H} \\
\text{H} \\
\text{H} \\
\text{H} \\
\text{H} \\
\end{array}} \quad \overset{+\text{H}}{\rightleftharpoons} \quad \overset{-\text{H}_2\text{O}}{\text{enamine}} \\
\text{nitroolefins 2a-g} & \quad \overset{\text{Pro-1}}{\begin{array}{c}
\text{4-OT} \\
\text{O} \\
\text{H} \\
\text{H} \\
\text{H} \\
\text{H} \\
\text{H} \\
\end{array}} \quad \overset{+\text{H}}{\rightleftharpoons} \quad \overset{-\text{H}_2\text{O}}{\text{enamine}} \\
\gamma\text{-nitroaldehydes 3a-g} & \quad \overset{\text{Pro-1}}{\begin{array}{c}
\text{4-OT} \\
\text{O} \\
\text{H} \\
\text{H} \\
\text{H} \\
\text{H} \\
\text{H} \\
\end{array}} \quad \overset{+\text{H}}{\rightleftharpoons} \quad \overset{-\text{H}_2\text{O}}{\text{enamine}} \\
\end{align*}
\]

2a and 3a \( R = 3\text{-o-PentO}-4\text{-MeO-C}_6\text{H}_3 \)
2b and 3b \( R = 3\text{-OH-4-MeO-C}_6\text{H}_3 \)
2c and 3c \( R \) isobutyl
2d and 3d \( R = \text{PhCH}=\text{CH} \)
2e and 3e \( R = \text{p(Cl)-C}_6\text{H}_4 \)
2f and 3f \( R = \text{p(F)-C}_6\text{H}_4 \)
2g and 3g \( R = \text{Ph} \)

Scheme S1. Proposed mechanism for the 4-OT-catalyzed Michael-type addition of acetaldehyde 1 to nitroolefins 2a-g yielding \( \gamma \)-nitroaldehydes 3a-g.

12. Absolute configuration determination of major enantiomers of enzymatically obtained 3a-g

The absolute configurations of the major enantiomers of enzymatically obtained 3a-g, respectively, were determined by HPLC with a chiral stationary phase and/or by optical rotation (Table S4). As described in detail in Section 9, the enantiomeric excesses of enzymatically obtained 3a-g were determined by HPLC or GC with a chiral stationary phase. The absolute configurations of the major enantiomers of products 3a and 3g, respectively, were both assigned to be (S) unambiguously by comparison with HPLC data in the literature (Table S4).

Optical rotations of enzymatically obtained 3a-g were measured to elucidate the absolute configurations of the major enantiomers of 3b-f, respectively. In all cases a negative rotation was found (Table S4). Comparison with literature data revealed that the chiral centers of the major enantiomers of 3a,c,g, respectively, all have the geometry as depicted in Scheme
S1. This means that major enantiomers of 3a,d-g have the (S)-configuration while the major enantiomer of 3c has the (R)-configuration. The optical rotation experiments thus confirmed the (S)-configuration of 3a and 3g already established with chiral HPLC. The deviant configuration of 3c is due to different prioritization of the R side chain as compared to 3a,b,d-g. The negative rotation of 3b could not be compared with literature data since, to the best of our knowledge, 3b has not been reported in the literature so far. Since 3b gave a negative rotation, like 3a,c-g did, we assume an identical geometry of the chiral center of 3b (Scheme S1) as compared to the geometry of the chiral centers of 3a,c-g.

References

2. Reference rotation not found in the literature. Geometry of chiral center of the major enantiomer of 3b was assumed to be identical as compared to the chiral centers of the major enantiomers of 3a,c-g, respectively, on basis of identical signs of rotation.


