Promiscuous Catalysis of the Asymmetric Michael-Type Addition of Linear Aldehydes to β-Nitrostyrene by the Proline-Based Enzyme 4-Oxalocrotonate Tautomerase

\[ \text{Yield up to } 92\% \text{ or up to } 93.7 \text{ ee (syn isomer) up to } 81\% \]

\[ R = \text{H, Me, Et, nPr, nBu, nPent, nHex} \]

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Abstract
Exploiting Catalytic Promiscuity
The proline-based enzyme 4-oxalocrotonate tautomerase (4-OT) promiscuously catalyzes the asymmetric Michael-type addition of linear aldehydes, ranging from acetaldehyde to octanal, to \textit{trans}-β-nitrostyrene in aqueous solvent. The presence of 1.4 mol\% of 4-OT effected formation of the anticipated γ-nitroaldehydes in fair to good yields with $dr$ values up to 93:7 and $ee$ values up to 81\%.
Introduction

The Michael addition reaction is widely used in organic synthesis for carbon-carbon bond formation.[1] In particular, the asymmetric Michael-type addition of unmodified aldehyde and ketone donors to nitroolefin acceptors[2] gives convenient access to γ-nitroaldehydes, which are versatile precursors[3] for various pharmacologically active compounds such as GABA receptor agonists. This type of Michael addition reaction has become feasible by the development of peptide- and L-proline-based organocatalysts.[2, 4] The catalytic amine moiety of these catalysts reacts with the carbonyl substrate to give nucleophilic enamine intermediates. Addition of the intermediate to the double bond of the nitroolefin results in the Michael-addition product, which is released from the catalyst by hydrolysis. The majority of organocatalytic methodologies for Michael-type additions involving aldehyde donors and nitroolefin acceptors, however, requires organic solvents.[2, 4, 5] Furthermore, examples of the controlled addition of acetaldehyde (1a), the ‘simplest’ of enolizable aldehydes but most difficult to control due to its relatively high reactivity, are not abundant and typically require 10-20 mol% of catalyst.[6, 7] Hence, there is interest in the development of alternative carbon-carbon bond formation methodologies that provide simple and environmentally friendly access to a large range of valuable chiral γ-nitroaldehydes.

Recently, we published a biocatalytic methodology for the asymmetric Michael-type addition of acetaldehyde (1a) to trans-nitrostyrene (2) (Scheme 1).[8] The enzyme 4-oxalocrotonate tautomerase (4-OT),[9] which carries an amino-terminal proline (Pro-1), promiscuously catalyzes the Michael-type addition of 1a to 2 to give product 3a with 89% ee.[8] The catalytic mechanism most likely involves formation of an enamine intermediate of 1a with Pro-1 (pKₐ ~6.4) of 4-OT (Scheme S1 in the Supporting Information),[8, 10] reminiscent of proline-based organocatalysis.[2, 4] NaCNBH₃ trapping and mass spectrometry experiments have shown that 1a exclusively reacts with the Pro-1 residue of 4-OT.[10a] The formed iminium ion is deprotonated resulting in an enamine intermediate. Reaction of this nucleophilic intermediate with the electrophilic double bond of 2 results in the carbon-carbon bond-forming Michael-type addition. Two other key catalytic residues are Arg-11 and Arg-39. It has been proposed that Arg-11 ensures correct substrate binding by coordinating the nitro-functionality of 2, while Arg-39 may act as a general acid catalyst.

**Scheme 1.** Asymmetric Michael-type addition of linear aldehydes 1a-g to trans-nitrostyrene (2) catalyzed by 4-OT.
delivering a proton at Cα of 2. The final product (3a) is released from 4-OT’s Pro-1 residue by hydrolysis (Scheme S1, Supporting Information).

We here report on the extension of our biocatalytic strategy by showing that 4-OT, in addition to acetaldehyde, also accepts the linear aldehydes propanal to octanal (1b-g) as donors for addition to 2 (Scheme 1). To the best of our knowledge this is the first biocatalytic methodology for the Michael-type addition of acetaldehyde up to octanal (C2-C8, 1a-g) to trans-nitrostyrene (2) in aqueous solvent. Notably, promiscuous enzyme-catalyzed asymmetric Michael additions are rare and most of them proceed in organic solvents and with low or moderate stereocontrol.

Results and discussion

Our previous examination of the 4-OT-catalyzed (0.7 mol%) Michael-type addition of 1a to 2 in 20 mM NaH2PO4 buffer (10% EtOH, v/v) at pH 7.3 revealed formation of undefinable side products lowering the yield of desired 3a to 46%. To eliminate these side reactions we lowered the pH of the reaction medium from 7.3 to 5.5 and raised the enzyme concentration from 0.7 to 1.4 mol%. To be able to verify by 1H NMR spectroscopy whether the 4-OT-catalyzed reaction of 2 with 1a under these new conditions proceeds more selectively towards 3a as compared to previously reported reaction conditions at pH 7.3, a preparative scale reaction with 1a (50 mM), 2 (2 mM, 18.9 mg) and 4-OT (27.6 µM, 1.4 mol%) in buffer (20 mM NaH2PO4/10% EtOH v/v, 60 mL, pH 5.5) was performed. The reaction was completed within 2 h and a standard work up was carried out according to procedures reported previously (see Supporting Information). Hardly any side products were observed and Michael adduct 3a was obtained in 70% yield (17.1 mg) with 81% ee in favor of the (S)-enantiomer (Table 1, entry 2). Finally, the apparent kinetic parameters of the 4-OT-catalyzed addition of 1a to 2 were estimated at pH 5.5 at a fixed concentration (50 mM) of 1a and varying concentrations (0.2–1.5 mM) of 2. By comparing the kinetic data obtained at pH 5.5 (k_{cat} = 7 × 10^{-2} s^{-1}, K_m = 1.1 mM) to the previously reported values at pH 7.3, we noticed that the catalytic efficiency at pH 5.5 (k_{cat}/K_m = 66 M^{-1} s^{-1}) remained identical to that at pH 7.3 (k_{cat}/K_m = 68 M^{-1} s^{-1}). Given that Pro-1 in 4-OT has a pK_a of ~6.4, this finding was surprising because lowering the pH from 7.3 to 5.5 would decrease the concentration of enzyme with Pro-1 in the correct protonation state (neutral form) to function as a nucleophile. However, reaction of Pro-1 with 1a would shift the equilibrium (from a cationic to a neutral form of Pro-1) and place more of the enzyme in the reactive state. In this manner, all of the enzyme could react with 1a and contribute to catalysis at pH 5.5. Inhibition and mass spectrometry experiments have shown rapid covalent labeling of Pro-1 by 1a, indicating that enamine formation is a fast process. Concluding up to here, running the 4-OT-catalyzed Michael-type addition of 1a to 2 at pH 5.5 gave considerable improvements as compared to pH 7.3 (Table 1, entries 1 and 2) since there was practically no formation of side products and Michael adduct 3a was obtained with a higher yield (70% instead of 46%), while the enantioselectivity and catalytic efficiency were conserved.
Next, we used a series of linear aliphatic aldehydes, propanal to octanal (1b-g), in combination with 2 to examine the donor scope of the 4-OT-catalyzed Michael-type addition reaction (Scheme 1). The analytical scale experiments were performed with 10 or 50 mM of aldehyde, 1 mM of 2 and 4-OT (69 µM) in buffer (20 mM NaH$_2$PO$_4$/10% EtOH v/v, pH 5.5), and reactions were monitored by UV spectroscopy ($\lambda_{\text{max}}$ of 2 = 320 nm). Aldehydes 1b and c were applied in 50 mM concentrations whereas 10 mM of aldehydes 1d-g was used because of their low solubility in water as compared to 1b and 1c. Incubation of propanal (1b) and 2 in the presence of 4-OT resulted in a decrease in the absorbance at 320 nm ($A_{320}$), showing almost complete depletion of 2 within 40 min (Figure 1C). Incubation of 1b and 2 without 4-OT did not show a significant decrease in the $A_{320}$ value within the same time frame, demonstrating that the conversion of 2 was enzyme-catalyzed (Figure 1A). Incubation of 2 with 4-OT in the absence of 1b showed only a slight decrease in the $A_{320}$ value within 40 min (Figure 1B), indicating that 1b is involved in the 4-OT-catalyzed conversion of 2 (Figure 1C). Although the 4-OT sample used in the assay was highly purified, chemically synthesized 4-OT$^{[12]}$ was tested to eliminate the possibility that any contaminating proteins from the expression strain are responsible for the observed

![UV spectra](image-url)
Michael-type addition activity. Indeed, a similar decrease in the $A_{320}$ value was observed when incubating 1b and 2 with folded synthetic 4-OT (69 µM) as with recombinant 4-OT (Figure 1D), showing that 4-OT is responsible for catalysis. In addition, incubation of the 4-OT P1A mutant with 1b and 2 showed only a slight decrease in the $A_{320}$ value, indicating that Pro-1 is crucial for this enzymatic activity (Figure S1A in the Supporting Information).

Linear aldehydes 1c-g were also successfully employed as donor substrates. The initial rates of the 4-OT-catalyzed conversions of 2 tend to decrease with increasing size of the aldehydes (Figure S2 in the Supporting Information). The addition reactions are also catalyzed by folded synthetic 4-OT, while an ignorable decrease of the $A_{320}$ value was observed by UV spectroscopy for the addition reactions without enzyme (Figures S3-S8 in the Supporting Information) or with the 4-OT P1A mutant (data not shown), showing that all these aldehyde additions to 2 are indeed enzyme-catalyzed. Furthermore, no significant decrease of the $A_{320}$ value was observed when 2 and hexanal (1e) were incubated with heat-denatured recombinant 4-OT (Figure S1B) or when 2 and propanal (1b) were incubated with unfolded synthetic 4-OT (Figure S1C). This indicates that the active site composition of 4-OT, and not solely a linear peptide with an N-terminal proline residue, is a requisite for catalytic activity. Interestingly, aceton and branched aldehydes, such as isobutanal, are not accepted by 4-OT for reaction with 2.

The 4-OT-catalyzed Michael-type additions of aldehydes 1b-e to 2 were carried out at preparative scale to identify the products and confirm that they are the result of Michael-type addition reactions. The reactions of 1b-c with 2 (Table 1, entries 3 and 4) were performed under the same conditions as those of the preparative scale addition of 1a to 2 described above (Table 1, entry 2). In the preparative scale reactions of 1d-e with 2 (Table 1, entries 5 and 6), 10 equivalents of aldehydes were applied instead of 25 equivalents because of their low solubility in water. After complete depletion of 2, monitored by UV spectroscopy, a standard work up procedure was performed to isolate products 3b-e (See Supporting Information). Product identity and diastereomeric ratios (dr) were determined by $^1$H NMR spectroscopy of the crude reaction mixture.$^{[4a, 4d, 4e]}$ Products 3b-e were purified by column chromatography and subsequently analyzed by HPLC on a chiral stationary phase to determine the enantiomeric excess (ee) of the major diastereoisomer. The absolute configurations were assigned by comparison with literature data (See Supporting Information).$^{[4a, 4d, 4e, 13]}$ The results confirmed that all four linear aldehydes 1b-e were accepted by 4-OT as donors in the Michael-type addition to 2. Michael adducts 3b-e were obtained with good to excellent syn selectivity (Table 1, entries 3-6), indicating that the size of the aldehydes employed has no impact on the diastereoselectivity. The observed syn selectivity is in accordance with Seebach’s model$^{[4a, 4c, 14]}$ for enamine addition to trans-nitrostyrene (2). The enantioselectivities and reaction rates tend to decrease with increasing length of the linear aldehydes. The yields with which products 3b-d were obtained are fair, however, a good yield of 92% was achieved (3e) by using an alternative work up procedure as compared to 3b-d (Table 1, entry 6 and see Supporting Information). Control experiments with aldehydes 1b-e and nitrostyrene (2), under identical conditions but without 4-OT,
Table 1. Preparative scale Michael-type addition reactions of linear aldehydes 1a-e to trans-nitrostyrene (2) catalyzed by 4-OT (1.4 mol%) at pH 5.5.

<table>
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<th>Substrate</th>
<th>t (h)</th>
<th>yield[a] (%)</th>
<th>dr[ab] (syn/anti)</th>
<th>ee[b] (%)</th>
<th>Product</th>
<th>Abs. conf[c]</th>
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<td>1a and 2</td>
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<td>46</td>
<td>-</td>
<td>89</td>
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<td>(3S)</td>
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<td>1a and 2</td>
<td>2</td>
<td>70</td>
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<td>81</td>
<td>3a</td>
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<td>93:7</td>
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<td>92[e]</td>
<td>93:7</td>
<td>23</td>
<td>3e</td>
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[a] Yields and dr's determined by 1H NMR of crude mixtures. [b] Determined by chiral HPLC analysis of the syn-isomer (see Supporting Information). [c] Absolute configuration of the major syn-enantiomer determined by comparison with literature data.[4a, 4d, 6, 14] [d] Data reported before at pH 7.3 and with 0.7 mol% of 4-OT.[8] [e] Determined using an alternative work up procedure as compared to 3a-d (see Supporting Information).

were performed. In all cases, no product formation (i.e. 3b-e) was observed by 1H NMR spectroscopy which ascertained that formation of 3b-e, respectively, in the presence of 4-OT is the result of enzyme-catalyzed Michael-type addition reactions.

4-OT is a member of the tautomerase superfamily,[9] a group of homologous proteins that share a characteristic β-α-β fold and a catalytic N-terminal proline residue. We investigated whether other members of this superfamily are capable of catalyzing the Michael-type addition reactions of 1a-g to 2. The superfamily members macrophage migration inhibitory factor (MIF)[9, 15] and YwhB,[9a] a 4-OT homologue from Bacillus subtilis, were chosen to test their ability to promiscuously catalyze the addition of 1a-g to 2. Indeed, the results from analytical scale reactions demonstrated that both enzymes can promiscuously catalyze these Michael-type addition reactions, although the catalytic activities were lower when compared to those of 4-OT (Figure S2 in the Supporting Information).

Conclusion

In conclusion, we have demonstrated that 4-OT accepts linear aldehydes ranging from acetaldehyde up to octanal (1a-g) as donor substrates and promiscuously catalyzes their asymmetric Michael-type addition to trans-nitrostyrene (2) in aqueous solvent. Good diastereoselectivities and fair enantioselectivities were established. As general trends, increasing bulkiness of the aldehydes did not influence the diastereoselectivities but diminished enantioselectivities and slowed down reaction rates. We realize that this new approach for Michael-type additions of aldehydes 1a-g to trans-nitrostyrene (2) is still far from application. However, it does feature a number of aspects that unite into a so far unique methodology: the reactions 1) are enzyme-catalyzed; 2) proceed in aqueous reaction media; and 3) involve a donor range from acetaldehyde up to octanal. To obtain more insight into the mechanism of the reaction and the binding mode of the aldehyde donors and nitroolefin acceptor, we have initiated studies to solve crystal structures of
4-OT in complex with these unnatural substrates. If these studies are successful, the derived insights will direct us in how to engineer 4-OT to enhance catalytic activities (i.e. increase reaction rates), enlarge the substrate scope and improve stereoselectivities. Together with our efforts to optimize work-up and isolation procedures to improve yields, this may lead to a practical methodology, that provides simple and environmentally friendly access to various valuable chiral γ-nitroaldehydes.

Acknowledgement
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References


7. Reference 6b is the only example we found in the literature that reports an asymmetric Michael-type addition reaction of acetaldehyde to a nitroolefin (nitrostyrene) in water. 10 mol% of organocatalyst yielded 13% of Michael-addition product after 18 h of reaction time. This methodology does not include any other aldehyde donor.


Supporting information

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1. General information

Materials
All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The sources for the buffers, solvents, components of Luria-Bertani (LB) media as well as the materials, enzymes and reagents used in molecular biology procedures are reported elsewhere. High purity synthetic 4-OT was obtained from GenScript USA Inc. (Piscataway, NY) and folded into the active homohexamer as described before. PCR purification, gel extraction and Miniprep kits were provided by Qiagen (Venlo, The Netherlands). Prepacked PD-10 sephadex G-25 columns were purchased from GE Healthcare Bio-science AB (Uppsala, Sweden). Ni-NTA sepharose was obtained from Qiagen. Oligonucleotides for DNA amplification were purchased from Operon Biotechnologies (Cologne, Germany).

General methods
Techniques for digestions with restriction enzymes, ligation, transformation and other standard molecular biology manipulations were based on methods described elsewhere. PCR was carried out using a DNA thermal cycler (model GS-1) purchased from Biolegio (Nijmegen, The Netherlands). The DNA sequencing was performed by Macrogen (Seoul, Korea). 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. Enzymatic assays were monitored using a V-650 or V-660 spectrophotometer purchased from Jasco (IJsselstein, The Netherlands). Kinetic data were analyzed by nonlinear regression data analysis using the Grafit program (Erithacus, Software Ltd., Horley, U.K.) obtained from Sigma Chemical Co. 1H NMR spectra were recorded on a Varian Inova 500 (500 MHz) or a Varian Mercury 200 (200 MHz) spectrometer. Chemical shifts for protons are reported in parts per million scale (δ scale) and are referenced to CHCl₃ (δ = 7.26). Reverse phase HPLC was carried out using an in-house analytical HPLC
equipped with a Shimazu LC-10 AT pump and a Shimazu SPD-M10A diode array detector using a Daicel Chiralpak AD-RH column. The HPLC chromatographic data were analyzed by data processing software (LC Solutions) obtained from Shimazu.

2. Expression and purification of proteins

*Expression and purification of 4-OT*

The 4-OT enzyme and its P1A mutant were produced in *E. coli* BL21(DE3) as native proteins without His-tag using the pET20b(+) expression system as described before.\(^5\) The construction of the expression vectors and the purification procedure for 4-OT and the mutant enzyme were reported previously.\(^5\)

*Expression and purification of His-tagged MIF*

The construction of the expression vector for MIF has been reported previously.\(^6\) The MIF protein (with a C-terminal His-tag) was produced in *E. coli* BL21(DE3) using the pET20b(+) expression system. LB medium (10 mL) containing ampicillin (Ap, 100 µg/mL) was inoculated with BL21(DE3) cells containing the appropriate expression plasmid. After overnight growth at 37°C, this culture was used to inoculate fresh LB/Ap medium (1 L) in a 5 L Erlenmeyer flask. Cultures were grown for 16–18 h at 37°C with vigorous shaking. Cells were harvested by centrifugation (5000 g, 15 min) and stored at -20°C before further use. The purification of His-tagged MIF was carried out following a protocol described before.\(^6\) The purified protein was flash frozen in liquid nitrogen and stored at -80°C until further use.

*Construction of the expression vector for His-tagged YwhB*

The YwhB gene was amplified from genomic DNA of *B. subtilis* using two synthetic primers by PCR. The forward primer 5′- CAG CGA **CAT** ATG CCA TAC GTA ACT GTC AAA ATG CTC -3′ contains an *NdeI* restriction site (in bold) followed by 24 bases corresponding to the coding sequence of the YwhB gene. To clone the YwhB gene in frame with the polyhistidine encoding region of the expression vector pET20b(+), the reverse primer 5′- GCA CTG CTC GAG TTC CAT ATC GCT CAG GCG TTT GCC TGC -3′, which contains an *XhoI* restriction site (in bold), was used. Amplification of the YwhB gene was carried out using the two synthetic primers, genomic DNA from *B. subtilis* as the template DNA, and the Phusion DNA polymerase following the protocol supplied with the polymerase (Finnzymes, Espoo, Finland). The resulting PCR product and the pET20b(+) vector were digested with *NdeI* and *XhoI* restriction enzymes, purified, and ligated using T4 DNA ligase. An aliquot of the ligation mixture was transformed into *E. coli* XL-1 Blue cells. Transformants were selected on LB/Ap plates and grown overnight at 37°C. Plasmid DNA was isolated from several colonies and analyzed by restriction analysis for the presence of the insert. The cloned YwhB gene was sequenced to verify that no mutations had been introduced during the amplification of the gene. The newly constructed expression vector was named pET20b(YwhB-His).
Overexpression and purification of His-tagged YwhB

The YwhB protein (with a C-terminal His-tag) was produced in *E. coli* BL21(DE3). *E. coli* BL21(DE3) cells containing the pET20b(YwhB-His) expression plasmid were collected from a LB/Ap plate using a sterile loop and used to inoculate 10 mL LB/Ap medium. After growth for 16 h at 37 °C, this culture was used to inoculate 1 L fresh LB/Ap medium in a 5 L Erlenmeyer flask. The culture was then grown for 16-18 h at 37°C with vigorous shaking. Cells were harvested by centrifugation (6,000 × g, 15 min) and suspended in lysis buffer (10 mL, 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). Cells were disrupted by sonication for 8 × 0.5 min (with 1-2 min rest in between each cycle) at a 60 W output. Unbroken cells and debris were removed by centrifugation (10,000 × g, 45 min). The supernatant was filtered through a membrane filter with 0.45 µM pore diameter. The flow-through was incubated with Ni-NTA (1 mL slurry in a small column at 4°C for 18 h), which had been equilibrated with lysis buffer in advance. The non-bound proteins were eluted from the column by gravity flow. The column was first washed with lysis buffer (2 × 10 mL) and then washed with buffer A (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0; 20 mL). Retained proteins were eluted with buffer B (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0; 3 mL). Fractions were analyzed by SDS-PAGE on gels containing acrylamide (10%), and those fractions containing YwhB protein were combined. The buffer was exchanged against 20 mM phosphate buffer (pH 7.3) using a prepacked PD-10 sephadex G-25 gel filtration column. The purified protein was flash frozen in liquid nitrogen and stored at ~80°C until further use.

3. General procedures for 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 25°C by following the decrease in absorbance at 320 nm, which corresponds to the depletion of *trans*-nitrostyrene (2). An aliquot of enzyme (4-OT, YwhB or MIF) was incubated in a 1 mm cuvette containing 20 mM NaH₂PO₄ buffer (pH 5.5), resulting in a final enzyme concentration of 69 µM (79 µM for MIF). Stock solutions of aldehydes (1a-g, separate stocks) were prepared in 20 mM NaH₂PO₄ buffer (pH 5.5), while a stock solution of 2 (10 mM) was prepared in absolute ethanol. The assay was initiated by the addition of an aldehyde (50 mM for aldehydes 1a-c and 10 mM for aldehydes 1d-g because of their low water solubility) and 2 (with a final concentration of 1 mM). The total volume of the reaction mixture was 0.3 mL and the ratio of water/ethanol was 9:1. The reactions were monitored for 1 h and the spectra were analyzed using the program provided by Jasco. Absorbance spectra of these experiments and various control experiments were recorded from 200 to 400 nm and are summarized in Figures S3-S8.

For control experiments with heat-denatured 4-OT, purified recombinant 4-OT was denatured by incubation at 100°C for 10 min. The denatured 4-OT was obtained by centrifugation, resuspended in phosphate buffer, and used in the UV-spectroscopic assay to monitor the
addition of hexanal (1e) to trans-nitrostyrene (2) (Figure S1B). The same reaction catalyzed by a corresponding amount of folded recombinant 4-OT was monitored as positive control.

For control experiments with unfolded synthetic 4-OT, unfolded synthetic 4-OT (1 mg) was incubated with 50 mM propanal (1b) and 1 mM trans-nitrostyrene (2) in a 25 ml round flask with gentle stirring. The total volume of the reaction mixture was 3 ml and the ratio of water/ethanol was 95/5. The same reaction catalyzed by a corresponding amount of fully folded synthetic 4-OT was monitored as positive control. At different time points, samples were withdrawn from the reaction mixture and analyzed by UV spectroscopy to establish the progress of the reaction (Figure S1C).

The initial reaction rates were determined by duplo experiments (as described above) and following the decrease in absorbance at 320 nm during time course UV measurements (λ_{max} of trans-nitrostyrene (2) = 320 nm). Rates are summarized in Figure S2.

**Kinetic assay**

The kinetic assay was performed with 50 mM acetaldehyde (1a), 29.4 µM 4-OT and varying concentrations (0.2 – 1.5 mM) of trans-nitrostyrene (2) as reported before.7

**General procedure for preparative scale reactions**

A stock solution of trans-nitrostyrene (2) (18 mg, 20 mM) was prepared in 6 mL absolute ethanol. The stock solution was slowly added to 20 mM NaH_{2}PO_{4} (pH 5.5) buffer containing 4-OT (11.3 mg, 1.4 mol%) in a 150 mL round flask. The reaction was initiated by the addition of aldehyde (from a stock solution in 20 mM NaH_{2}PO_{4} buffer, pH 5.5) and the total volume of the reaction mixture was 60 mL. The final concentration of acetaldehyde (1a), propanal (1b) and butanal (1c) was 50 mM, whereas the final concentration of pentanal (1d) and hexanal (1e) was 20 mM because of their low solubility in water. The reaction mixture was incubated at room temperature for 2–8.5 h and the progress was monitored by UV-spectroscopy. After complete conversion of 2, 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 unless otherwise stated. The flow through was collected and extracted with diethyl ether (3 × 40 mL). The combined organic layers were dried over MgSO_{4} and concentrated in vacuo.

In the case of preparative scale reaction of 1e and 2, the reaction mixture was directly extracted with diethyl ether (3 × 40 mL) without removing the enzyme using a Vivaspin column. The combined organic layers were filtered to remove the precipitated enzyme and then concentrated in vacuo after drying. The resulting product mixture containing 3e was analyzed by polyacrylamide gel electrophoresis (PAGE) using a sodium dodecyl sulfate (SDS) gel containing polyacrylamide (10%), and no protein was detected.

In all cases (3a-e), a 1H NMR spectrum recorded from the residue showed complete conversion of 2 and the yield and diastereomeric ratio (dr) of the nitroaldehyde product were determined. Trace amounts of self-aldolization products of aldehydes 3b-3e were also observed. The crude mixtures containing 3b-d, respectively, were purified by column
chromatography (silica gel, Heptane/AcOEt 8:1, visualization: KMnO₄) to afford Michael adducts 3b-d. The separation of product 3e from the self-aldolization product of 1e was not successful by column chromatography. The purified products 3b-d and crude mixture containing 3e were used for the enantiomeric excess (ee) determination by chiral HPLC. Blank reactions of aldehydes (1b-e) and 2 under the same conditions, but without enzyme, were performed as control experiments. In all cases, no product formation (i.e. 3b-e) was observed by ¹H NMR spectroscopy after the same work-up procedure as used for the 4-OT-catalyzed reactions (described above).

4. Characterization of products

Assignment of the stereoisomers was performed by comparison with ¹H NMR spectroscopic data in the literature. The absolute stereochemistry of products 3a-e was determined by comparison of their retention times on chiral phase HPLC with those reported in literature.

(S)-4-Nitro-3-phenylbutanal (3a)

Product 3a was prepared from acetaldehyde (1a) and trans-β-nitrostyrene (2) according to the general procedure for the preparative Michael-type addition reactions. The ¹H NMR spectroscopic data are in agreement with published data. To determine the enantiomeric excess, 3a was converted into the corresponding ethylene glycol acetal and purified as reported previously. HPLC was performed using a Chiracel OD column (n-heptane/i-PrOH 90:10, 40°C) at 1 mL/min, UV detection at 210 nm: tᵣ: (minor) = 13.0 min, (major) = 15.4 min. The HPLC data are in accordance with the literature.

(2R,3S)-2-Methyl-4-nitro-3-phenylbutanal (3b)

Product 3b was prepared from n-propanal (1b) and trans-β-nitrostyrene (2) according to the general procedure for the preparative scale Michael-type addition reactions. The ¹H NMR spectroscopic data are in agreement with published data. The enantiomeric excess was determined by HPLC according to a literature method using a Chiracel OD-H column (n-heptane/i-PrOH 90:10, 25°C) at 1 mL/min, UV detection at 210 nm: tᵣ: (syn, minor) = 23.8 min, (syn, major) = 34.5 min. The HPLC data are in accordance with the literature.
(2R,3S)-2-Ethyl-4-nitro-3-phenylbutanal (3c)

Product 3c was obtained from n-butanal (1c) and trans-β-nitrostyrene (2) according to the general procedure for the preparative scale Michael-type addition reactions. The 1H NMR spectroscopic data are in agreement with published data.8,9,11 The enantiomeric excess was determined by HPLC according to literature using a Chiracel AD column (n-heptane/i-PrOH 99.5:0.5, 25°C) at 0.9 mL/min, UV detection at 210 nm: t_R: (syn, major) = 34.4 min, (syn, minor) = 46.2 min. The HPLC data are in accordance with the literature.8,9,11

(2R,3S)-2-Propyl-4-nitro-3-phenylbutanal (3d)

Product 3d was obtained from n-pentanal (1d) and trans-β-nitrostyrene (2) according to the general procedure for the preparative scale Michael-type addition reactions. The 1H NMR spectroscopic data are in agreement with published data.8,10,11 The enantiomeric excess was determined according to literature by HPLC using a Chiracel OD-H column (n-heptane/i-PrOH 85:15, 40°C) at 0.8 mL/min, UV detection at 210 nm: t_R: (syn, minor) = 21.2 min, (syn, major) = 27.8 min. The HPLC data are in accordance with the literature.8,10,11

(2R,3S)-2-Butyl-4-nitro-3-phenylbutanal (3e)

Product 3e was obtained from n-hexanal (1e) and trans-β-nitrostyrene (2) according to the general procedure for the preparative scale Michael-type addition reactions. The 1H NMR spectroscopic data are in agreement with published data.8,9,11 The enantiomeric excess was determined according to literature by HPLC using a Chiracel OD-H column (n-heptane/i-PrOH 80:20, 25°C) at 1.0 mL/min, UV detection at 210 nm: t_R: (syn, minor) = 10.1 min, (syn, major) = 12.8 min. The HPLC data are in accordance with the literature.8,9,11
Reverse phase HPLC data

The enantiomeric excess (ee) of the syn-isomer of 3a-e was also determined using a reverse phase HPLC method we developed in our laboratories. The results are in accordance with those obtained from normal phase HPLC and the data are summarized in Table S1.

5. General procedures for synthesis of racemic compounds

Racemic 4-nitro-3-phenylbutanal (rac-3a) was synthesized according to a literature procedure. Other racemic compounds (rac-3b-e) were synthesized based on the following procedure: trans-nitrostyrene (2, 100 mg, 0.67 mmol) and DL-proline (15.4 mg, 0.13 mmol, 20 mol%) were dissolved in tetrahydrofuran (3 mL). The solution was stirred at room temperature under nitrogen atmosphere. The reactions were initiated by the addition of aldehyde (1b-e, 3.33 mmol), and the reaction progress was monitored by thin layer chromatography (TLC, silica, n-heptane/EtOAc 4:1, Rf nitrostyrene = 0.5, Rf product = 0.2-0.25, visualization: KMnO4). After 2 was completely converted, the solvent was evaporated and the residue was purified by column chromatography (silica gel, n-heptane/AcOEt 8:1). 1H NMR spectroscopic data of 3b-e were in accordance with the literature.
6. Table and figures

Table S1. Reverse phase HPLC data of nitroaldehydes 3a-e obtained with a Daicel Chiralpak AD-RH column.

<table>
<thead>
<tr>
<th>Product</th>
<th>Eluent</th>
<th>Flow rate (mL/min)</th>
<th>$t_R^{[1]}$ (min)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a$^{[b]}$</td>
<td>MeCN:Water (70/30)</td>
<td>0.5</td>
<td>8.3, 11.0 (major)</td>
<td>83</td>
</tr>
<tr>
<td>3b</td>
<td>MeCN:Water (40/60)</td>
<td>0.5</td>
<td>17.7 (major), 20.3</td>
<td>50</td>
</tr>
<tr>
<td>3c</td>
<td>MeCN:Water (40/60)</td>
<td>0.5</td>
<td>24.2 (major), 31.4</td>
<td>32</td>
</tr>
<tr>
<td>3d</td>
<td>MeCN:Water (35/75)</td>
<td>0.5</td>
<td>61.0 (major)$^{[c]}$, 78.0</td>
<td>55</td>
</tr>
<tr>
<td>3e</td>
<td>MeCN:Water (40/60)</td>
<td>0.5</td>
<td>47.7 (major), 57.0</td>
<td>20</td>
</tr>
</tbody>
</table>

[a] Retention times of the syn-isomers. [b] Compound 3a was converted into the corresponding ethylene glycol acetal$^{11,12}$ for ee determination. [c] In the HPLC chromatogram of the mixture of all four stereoisomers of 3d, the signal of the major syn-isomer ($t_R = 61.0$) overlaps with the signal of the major anti-isomer ($t_R = 61.0$). However, on basis of $^1$H NMR spectra and HPLC chromatograms of the reference mixture of 3d (see paragraph 5 above) and the enzymatically obtained mixture of 3d, the ee could be determined.

Figure S1. A) UV spectra following the depletion of 1 mM trans-nitrostyrene (2) in the presence of 50 mM propanal (1b) and recombinant 4-OT (rec-4-OT, 69 µM), 4-OT P1A (69 µM), or without 4-OT (Blank); B) UV spectra following the depletion of 1 mM trans-nitrostyrene (2) in the presence of 10 mM hexanal (1e) and recombinant 4-OT (69 µM), denatured 4-OT, or without 4-OT (Blank); C) UV spectra following the depletion of 1 mM trans-nitrostyrene (2) in the presence of 50 mM propanal (1b) and fully folded synthetic 4-OT (syn-4-OT, folded, 49 µM), a corresponding amount of unfolded synthetic 4-OT (syn-4-OT, unfolded, 1 mg), or without synthetic 4-OT (Blank). For the incubation of 2 and 1b with unfolded synthetic 4-OT, the observed minor decrease in the $A_{320}$ value may be explained by the partial folding of synthetic 4-OT during the course of the experiment.
**Figure S2.** Overview of initial rates (µM/s) of Michael-type addition reactions of linear aldehydes (1a-1g) to trans-nitrostyrene (2) catalyzed by 4-OT, YwhB and MIF. For experimental details see the paragraph General procedure for UV-spectroscopic assays of analytical scale reactions.

**Figure S3.** UV spectra showing the depletion of trans-nitrostyrene (2) incubated in 20 mM Na$_2$HPO$_4$ buffer/10% EtOH v/v at pH 5.5 with A) acetaldehyde (1a), B) acetaldehyde (1a) and recombinant 4-OT (69 µM), C) acetaldehyde (1a) and synthetic 4-OT (69 µM).
Figure S4. UV spectra showing the depletion of trans-nitrostyrene (2) incubated in 20 mM Na2HPO4 buffer/10% EtOH v/v at pH 5.5 with A) butanal (1c), B) butanal (1c) and recombinant 4-OT (69 µM), C) butanal (1c) and synthetic 4-OT (104 µM).

Figure S5. UV spectra showing the depletion of trans-nitrostyrene (2) incubated in 20 mM Na2HPO4 buffer/10% EtOH v/v at pH 5.5 with A) pentanal (1d), B) pentanal (1d) and recombinant 4-OT (69 µM), C) pentanal (1d) and synthetic 4-OT (104 µM).
Figure S6. UV spectra showing the depletion of trans-nitrostyrene (2) incubated in 20 mM Na₂HPO₄ buffer/10% EtOH v/v at pH 5.5 with A) hexanal (1e), B) hexanal (1e) and recombinant 4-OT (69 µM), C) hexanal (1e) and synthetic 4-OT (104 µM).

Figure S7. UV spectra showing the depletion of trans-nitrostyrene (2) incubated in 20 mM Na₂HPO₄ buffer/10% EtOH v/v at pH 5.5 with A) heptanal (1f), B) heptanal (1f) and recombinant 4-OT (69 µM), C) heptanal (1f) and synthetic 4-OT (104 µM).
**Figure S8.** UV spectra showing the depletion of *trans*-nitrostyrene (2) incubated in 20 mM Na$_2$HPO$_4$ buffer/10% EtOH v/v at pH 5.5 with A) octanal (1g), B) octanal (1g) and recombinant 4-OT (69 µM), C) octanal (1g) and synthetic 4-OT (104 µM).

**Scheme S1.** Proposed mechanism for the 4-OT catalyzed addition of 1a to 2 to yield 3a.
7. References


