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Exploiting Catalytic Promiscuity for Biocatalysis

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Practical method: Asymmetric Michael-type Additions of Acetaldehyde to Nitroolefins Catalyzed by 4-Oxalocrotonate Tautomerase (4-OT) Yielding Valuable γ-Nitroaldehydes

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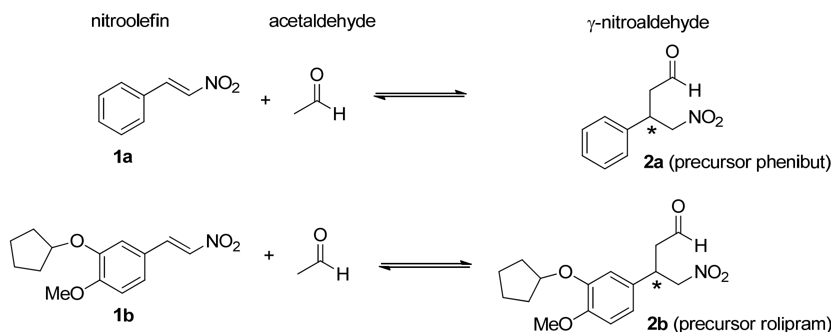
Introduction

γ-Nitroaldehydes are useful precursors for γ-aminobutyric acid (GABA) derivatives, several of which represent marketed pharmaceuticals.[1] The Michael-type addition of acetaldehyde to nitroolefins gives convenient access towards relevant chiral γ-nitroaldehydes (Scheme 1). The first successful methodology for this type of reaction was developed in the field of organocatalysis[2,3] and documented in the literature in 2008. We lately presented a biocatalytic procedure for the Michael-type addition of acetaldehyde to various nitroolefins, which involves the homohexameric enzyme 4-oxalocrotonate tautomerase (4-OT).[4,5,6] 4-OT is a member of the tautomerase superfamily and is part of a catabolic pathway for aromatic hydrocarbons in the soil bacterium *Pseudomonas putida* mt-2. 4-OT is a small enzyme (only 62 amino acid residues per monomer) and is characterized by a catalytic N-terminal proline residue (Pro-1) that resides in the active site.[7,8,9,10] We here describe the production and purification of the enzyme 4-OT, as well as its use as a catalyst in the Michael-type addition of acetaldehyde to nitroolefins **1a**-**b** to produce γ-nitroaldehydes **2a**-**b** (Scheme 1).[4,5,6]



Scheme 1. Michael-type addition of acetaldehyde to nitroolefins **1a**-**b** yielding chiral γ-nitroaldehydes **2a**-**b**. * = chiral center.

Materials and equipment for expression and purification of 4-OT

- (Conical-bottom) centrifuge tube (50 mL, CELLSTAR)
- Ammonium sulphate (3.2 M), 50 mL
- Ampicillin (100 mg/ml stock solution in water, filter sterilized)
- Centrifuge capable of reaching 15000 rpm with temperature control
- DEAE-sepharose (CL-6B, Sigma-Aldrich) column (10×1.0 cm filled with ~8 ml DEAE-sepharose resin)
- Dialysis membrane with MWCO 2000 Da (Spectrum labs)
- *E. coli* BL21(DE3) containing pET20b(4-OT) expression vector (frozen glycerol stock at −80°C)

- Erlenmeyer flask (3 L)
- Eppendorf tubes (1.5/2 mL)
- LB medium (1 L, sterilized)
- Magnetic stirrer
- PD-10 sephadex G-25 gel filtration column (GE Healthcare Bio-sciences AB)
- Phenyl-sepharose (CL-4B, Sigma-Aldrich) column (10×1.0 cm filled with ~8 ml phenyl-sepharose resin)
- Quartz cuvette with 1 mm or 10 mm light path (Hellma Analytics)
- Rotary shaker with temperature control
- Sodium dihydrogenphosphate (NaH_2PO_4, 20 mM, pH 7.3), 100 mL
- Sodium dihydrogenphosphate (NaH_2PO_4, 10 mM, pH 8.0) containing ammonium sulphate [$(NH_4)_2SO_4$, 1.6 M], 100 mL
- Sodium dihydrogenphosphate [NaH_2PO_4, 10 mM, pH 8.0] containing sodium sulphate [Na_2SO_4, 0.5 M], 100 mL
- Sodium dihydrogenphosphate [NaH_2PO_4, 10 mM, pH 8.0], (buffer A), 250 mL
- Sodium dodecyl sulfate (SDS) gels containing polyacrylamide (10%)
- Sterile loop
- Syringe filter, pore size 0.2 µm (FP30/0.2,Whatman)
- UV-VIS spectrophotometer
- Vortex mixer

Procedure for expression and purification of 4-OT

1. Using a sterile loop, cells from a frozen glycerol stock of *E. coli* BL21(DE3) harboring the pET20b(4-OT) expression vector were used to inoculate 5 mL LB medium, in a 50 mL conical-bottom centrifuge tube, containing ampicillin (100 µg/mL).
2. After overnight growth with shaking (250 rpm) at 37°C, this culture was used to inoculate the rest of LB medium (995 mL) containing ampicillin (100 µg/mL) in a 3 L Erlenmeyer flask. The culture was incubated overnight at 37°C with vigorous shaking (200 rpm) on a rotary shaker.
3. The culture was divided into two equal portions and cells were harvested by centrifugation (20 min at 4500 rpm) after an OD_{600} value of ~4.5 was reached.[11]
4. Cells harvested from 0.5 L of culture were resuspended in ~10 mL of buffer A by using a vortex mixer. [Optional: for storing the cells at −20°C, leave the cells in 10 mM NaH_2PO_4 buffer (pH 8.0, buffer A).]
5. The cell suspension was transferred to sonication tubes (centrifuge tubes) and sonicated at high power output for 8-10 min to disrupt the cells. After centrifugation (45 min at 15000 rpm), the soluble fraction (supernatant) containing 4-OT was separated from the insoluble part (pellet) [Note: Keep the cell suspension in a cooling bath (e.g. water/ice) during the sonication process to avoid heat inactivation of 4-OT.]

6. The supernatant that contains 4-OT was then loaded onto a DEAE-sepharose column, which had been previously equilibrated with buffer A, using gravity flow. The flow-through was discarded.
7. The column was first washed with 3×10 mL buffer A and then the protein was eluted by gravity flow using buffer A containing 0.5 M Na_2SO_4 (12 mL). Fractions (1.5 mL × 8) from the elution step were collected in Eppendorf tubes (1.5 mL) and the presence of 4-OT in each fraction was examined by SDS-PAGE.
8. The fractions containing 4-OT were then combined and mixed with $(NH_4)_2SO_4$ (final concentration: 1.6 M). After gentle stirring with a magnetic stir bar at 4°C for 2 h, the precipitate was pelleted by centrifugation (20 min at 13300 rpm, 4°C).
9. The supernatant was filtered (syringe filter with 0.2 µm pore size) and loaded onto a phenyl-sepharose column that had been previously equilibrated with buffer A containing 1.6 M $(NH_4)_2SO_4$.
10. The supernatant was left to flow through the column and the flow-through was discarded. The column was then washed with 3×10 mL of loading buffer [buffer A containing 1.6 M $(NH_4)_2SO_4$], after which the protein was eluted by gravity flow using buffer A (12 mL). Fractions of the wash step (~30 mL) and the elution step (~1.5 mL × 8) were analyzed by SDS-PAGE. [Note: wild-type 4-OT has little interaction with the phenyl-sepharose column and should therefore elute as homogenous protein (>95% purity as assessed by SDS-PAGE) in the wash step.]
11. Fractions that contained pure 4-OT were combined and the buffer was exchanged against 20 mM NaH_2PO_4 buffer (pH 7.3) using a pre-packed PD-10 sephadex G-25 gel filtration column. [Optional: An additional dialysis step is recommended to reduce the high salt concentration (1.6 M $(NH_4)_2SO_4$) in the sample before exchanging the buffer on a PD-10 column. In a typical dialysis procedure, the fractions containing pure 4-OT were combined and transferred into a dialysis membrane (MWCO 2000 Da). The dialysis membrane containing the sample was then placed in a 5 L beaker containing ~4 L 20 mM NaH_2PO_4 buffer (pH 7.3) with gentle stirring on a magnetic stirrer overnight.]
12. The concentration of purified 4-OT in 20 mM NaH_2PO_4 buffer (pH 7.3) was determined with a spectrophotometer using the method of Waddell.[12] A typical yield of purified 4-OT that can be isolated from 0.5 L of cell culture following this protocol is 25–50 mg.
13. The purified protein was divided into aliquots (100-200 µl) and stored at 4°C until further use. [Optional: Purified 4-OT can be stored for at least one month upon freezing the protein with liquid nitrogen and when kept at -80°C.]

Materials and equipment for synthesis of 2a

- *Trans*-β-nitrostyrene **1a** (18.0 mg, commercially available, Sigma-Aldrich)
- Acetaldehyde (132 mg, ~0.17 mL)
- EtOH (6 mL)
- 20 mM NaH_2PO_4 buffer, pH 5.5 (~54.0 mL)

- 4-OT (11.3 mg from a stock solution in 20 mM NaH_2PO_4 buffer, pH 7.3)
- Diethyl ether (120 mL)
- $MgSO_4$ (~0.5 g)
- Deuterated chloroform ($CDCl_3$, 0.65 mL for ^1H NMR analysis)
- Balance
- Measuring cylinder (50 mL)
- Glass flask (150 mL)
- Standard pipette for 4-OT addition
- 1 mL Syringe (NORM-JECT) for acetaldehyde addition
- UV-VIS spectrophotometer
- Cuvette (preferably path length = 1 mm, volume = 300 μL. See paragraph 'analytical methods')
- Two vivaspin columns (Sartorius Stedim Biotech S.A., France) with a 5000 Da molecular weight cut-off filter
- Separatory funnel (250 mL)
- Two Erlenmeyer flasks (100 mL and 250 mL)
- Magnetic stirrer
- Magnetic stir bar (for drying with $MgSO_4$ during work up procedure)
- Funnel with filter paper
- Round bottom glass flask (250 mL)
- Rotary evaporator connected to a vacuum pump
- NMR tube
- NMR spectrometer

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PRACTICAL METHODS FOR SYNTHESIZING Γ-NITROALDEHYDES

Procedure for synthesis of 2a (from reference 5)

1. In a glass flask (150 mL), a solution of nitroolefin **1a** (18.0 mg, 0.12 mmol) in EtOH (6.0 mL) was added to a mixture of acetaldehyde (132 mg, 3.0 mmol) and 4-OT (11.3 mg from a stock solution in 20 mM NaH_2PO_4 buffer pH 7.3, 1.7×10^{-3} mmol, 1.4 mol%) in 20 mM NaH_2PO_4 buffer, pH 5.5 (final volume of reaction mixture: 60 mL. Final ratio water/EtOH: 90/10 v/v).
2. The mixture was incubated at room temperature and reaction progress was monitored by recording UV-spectra of aliquots taken from the reaction mixture after regular time intervals. Note: to avoid inactivation of the enzyme, it is recommended not to stir the reaction mixture with a stirring magnet but rather to gently shake the mixture every 30 min.
3. After 2.0 h, all **1a** was converted. The reaction mixture was divided into two equal portions, transferred to two Vivaspin columns (Sartorius Stedim Biotech S.A., France) with a 5000 Da molecular weight cut-off filter, and centrifuged (4000 rpm, RT) to remove 4-OT.
4. The flow-throughs were collected, combined, and extracted with diethyl ether (3×40 mL). The combined organic layers were dried with $MgSO_4$, filtered, and concentrated

in vacuo with a rotary evaporator to yield **2a** with high purity (16.2 mg, 8.4×10^{-2} mmol, 70%) as a colorless oil.

5. The ^{1}H NMR spectroscopic data of **2a** were in agreement with published data.[13,14] Enantiomeric excess was determined by derivatization of the aldehyde moiety of **2a** into the corresponding cyclic acetal.[4] Normal phase HPLC analysis of derivatized **2a** using a Chiracel OD column revealed an enantiomeric excess of 81% in favor of the (*S*)-enantiomer. HPLC parameters: eluent: (*n*-heptane/*i*-PrOH 90:10, 40°C), flow rate: 1 mL/min, UV detection at 210 nm, t_R: (minor) = 13.0 min, (major) = 15.4 min. The HPLC data are in accordance with the literature.[14]

Materials and equipment for synthesis of 2b

- Nitroolefin **1b**: (*E*)-2-(cyclopentyloxy)-1-methoxy-4-(2-nitrovinyl)benzene (31.6 mg)[15]
- Acetaldehyde (132 mg, ~0.17 mL)
- DMSO (24 mL)
- 20 mM NaH_2PO_4 buffer, pH 5.5 (~36.0 mL)
- 4-OT (30 mg from stock solution in 20 mM NaH_2PO_4 buffer, pH 7.3)
- Water (18 mL)
- Chloroform (48 mL)
- $MgSO_4$ (~0.5 g)
- Deuterated chloroform ($CDCl_3$, 0.65 mL for ^{1}H NMR analysis)
- Balance
- Measuring cylinder (50 mL)
- Glass flask (150 mL)
- Standard pipette for 4-OT addition
- 1 mL Syringe (NORM-JECT) for acetaldehyde addition
- UV-VIS spectrophotometer
- Cuvette (preferably path length = 1 mm, volume = 300 μL. See paragraph 'analytical methods')
- Six CELLSTAR tubes (15 mL, polypropylene (PP), conical bottom)
- Acid-resistant CentriVap vacuum concentrator (Labconco, 78100 series) connected to a cold trap and a vacuum pump
- Separatory funnel (100 mL)
- Two Erlenmeyer flasks (25 mL and 100 mL)
- Magnetic stirrer
- Magnetic stir bar (for drying with $MgSO_4$ during work up procedure)
- Funnel with filter paper
- Round bottom glass flask (100 mL)
- Rotary evaporator connected to a vacuum pump
- NMR tube
- NMR spectrometer

Procedure for synthesis of 2b (from reference 6)

1. In a glass flask (150 mL), a solution of nitroolefin **1b** (31.6 mg, 0.12 mmol) in DMSO (6.0 mL) was added to a mixture of acetaldehyde (132 mg, 3.0 mmol) and 4-OT (30 mg from a stock solution in 20 mM NaH_2PO_4 buffer pH 7.3, 4.4×10^{-3} mmol, 3.7 mol%) in 20 mM NaH_2PO_4 buffer (pH 5.5) and DMSO (final volume of reaction mixture: 60.0 mL. Final ratio water/DMSO: 60/40 v/v).
2. Note: DMSO was required as a co-solvent to achieve sufficient solubility of nitroolefin **1b** and product **2b** in aqueous buffer. DMSO enhances solubility of **1b** and **2b**, does not impede catalytic activity of 4-OT, and does not chemically react with any of the reagents (see reference 6 for full explanation).
3. The mixture was incubated at room temperature and reaction progress was monitored by recording UV-spectra of aliquots taken from the reaction mixture after regular time intervals. Note: to avoid inactivation of the enzyme, it is recommended not to stir the reaction mixture with a stirring magnet but rather to gently shake the mixture every 30 min.
4. After 2.5 h, all **1b** 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).
5. 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 $MgSO_4$, filtered, and concentrated *in vacuo* with a rotary evaporator to yield **2b** with high purity (23.6 mg, 7.7×10^{-2} mmol, 64%) as a colorless oil.
6. The ^{1}H NMR spectroscopic data of **2b** were in agreement with published data.[2] Enantiomeric excess was determined by derivatization of the aldehyde moiety of **2b** into its corresponding methyl ester.[16] Normal phase HPLC analysis of derivatized **2b** using a Chiralpak IB column revealed an enantiomeric excess of 96% in favor of the (*S*)-enantiomer.[16] HPLC parameters: eluent: (*n*-heptane/*i*-PrOH 95:5, 25°C), flow rate: 1 mL/min, UV detection at 220 nm, t_R: (minor) = 32.2 min, (major) = 34.9 min.

Analytical methods

The biocatalytic conversions of nitroolefins **1a-b** into **2a-b** were monitored by UV spectroscopy. After regular time intervals (typically 30 min), 300 μL of reaction mixture was transferred into a 300 μL cuvette with a path length of 1 mm (see note below) and a UV-spectrum was recorded (200–500 nm). A decrease of absorbance at λ_{max} of **1a-b** indicates the depletion of **1a-b** ($\lambda_{max,\mathbf{1a}}$ = 320 nm, ε_{max} = 14.4 mM^{-1} cm^{-1}; $\lambda_{max,\mathbf{1b}}$ = 378 nm, ε_{max} = 13.5 mM^{-1} cm^{-1}). After recording the UV-spectrum, the contents of the cuvette were recombined with the reaction mixture (with a syringe). The work up procedure was initiated when the absorbance at λ_{max} of the nitroolefin (**1a**-**b**) had totally vanished which indicates full conversion of the nitroolefin (**1a**-**b**).

Note: obviously one may use a cuvette with a path length of 10 mm and volume of ~1 mL instead. However, one should then take an aliquot of 100 μL of the reaction mixture and dilute with 900 μL of buffer (20 mM NaH_2PO_4, pH 5.5).

Results and conclusion

The enzyme 4-OT can be readily produced and purified. About 50 to 100 mg of 4-OT can be obtained from 1 L of cell culture. The enzyme can be stored at −80°C in concentrations of 10 to 20 mg/mL (in 20 mM NaH_2PO_4 buffer, pH 7.3) for at least one month without significant loss of catalytic activity. 4-OT can be easily tested on whether it is fully catalytically active by assaying its natural tautomerase activity with the substrates 2-hydroxymuconate[17] or phenyl(enol)pyruvate[18] and comparing with literature data.

The enzyme 4-OT promiscuously catalyzes the Michael-type addition of acetaldehyde to nitroolefins **1a-b** yielding γ-nitroaldehydes **2a-b** (Scheme 1). The methodology is characterized by relatively low catalyst loading (compared to 10−20 mol% in organocatalysis),[2,3,19,20,21,22] high stereoselectivities, and aqueous reaction media (Table 1). Reaction times of 2.5 h or less were sufficient for full conversion of nitroolefins **1a-b**, respectively. Side products were hardly formed as revealed by straightforward work-up procedures without column chromatography which yielded **2a-b** with high purity (^{1}H NMR spectroscopy). Respective yields of 70% and 64% were established for **2a-b** while e.e. values of 81% and 96%, respectively, were found. Catalysis of the Michael-type additions takes place in the active site of 4-OT and its characteristic N-terminal proline is a key catalytic residue as demonstrated by various control experiments.[4,5,6] 4-OT fully retains its 'Michaelase' activity in aqueous solvent systems containing up to 50% DMSO (v/v).[6] This methodology is therefore not only restricted to water-soluble substrates but also allows application of poorly water-soluble chemicals such as **1b** since its solubility in aqueous solvent systems is greatly enhanced in the presence of DMSO. In addition to acetaldehyde and aromatic nitroolefins, 4-OT also accepts linear aldehydes up to octanal as donors[5] and aliphatic nitroolefins as acceptors[6] for Michael-type addition reactions.

Table 1. Conditions for, and results of, 4-OT-catalyzed Michael-type additions of acetaldehyde to nitroolefins **1a** and **1b** in 20 mM NaH_2PO_4 buffer (pH 5.5) and co-solvent yielding **2a** and **2b** (Scheme 1).

nitroolefin	4-OT (mol%)	co-solvent (v/v)	reaction time (h)	product	yield (%)	e.e. (%)
1a	1.4	EtOH 10%	2	**2a**	70	81
1b	3.7	DMSO 40%	2.5	**2b**	64	96

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