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

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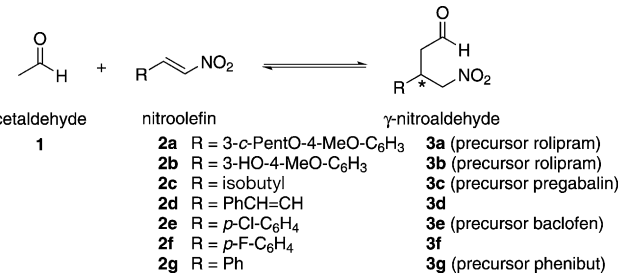
γ-Nitroaldehydes are versatile and practical precursors for chiral γ-aminobutyric acids (GABAs). In particular, prominent GABA analogues, such as marketed pharmaceuticals phenibut[1] ($GABA_B$ receptor agonist, anxiolytic), pregabalin[2] (anticonvulsant), baclofen[3] ($GABA_B$ receptor agonist, anti-alcoholism), 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 nitroolefins has recently become viable by the development of proline- and peptide-based organocatalysts.[7,8] However, examples including acetaldehyde as the 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 the donor and mainly exhibit low stereoselectivities.[10]



Scheme 1. Michael-type addition of acetaldehyde **1** to nitroolefins **2a–g**. * = chiral center.

We herein 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 (acetaldehyde up to octanal) to *trans*-nitrostyrene,[12,13] 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.[14] 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 **1** 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)[15] were incubated with acetaldehyde **1** (25–150 mM)[16] and 4-OT (32–150 μM)[16] in NaH_2PO_4 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 NaH_2PO_4 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%

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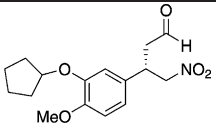
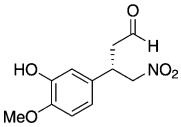
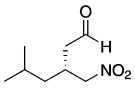
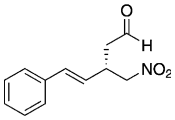
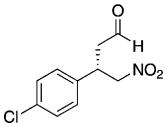
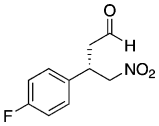
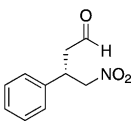
v/v) are suitable co-solvents that meet all above-mentioned criteria.[17]

The analytical scale reactions were followed by monitoring the change of absorbance at λ_{max} of **2a–f** by UV-spectroscopy.[18] During all reactions, decrease of the absorbance at $\lambda_{max,\mathbf{2a–f}}$ was observed in course of time (20–120 min),[17] which indicated almost complete depletion of nitroolefins **2a–f** (see Figures S1–S6 in the Supporting Information). Identical experiments with 4-OT and **2a–f**, respectively, but in the absence of acetaldehyde **1**, showed negligible decreases of absorbances at $\lambda_{max,\mathbf{2a–f}}$ (except for compound **2c**, vide infra), which demonstrated that acetaldehyde **1** is involved in the 4-OT-catalyzed conversions of **2a–f** (see 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** (see Figure 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 $\lambda_{max,\mathbf{2a–f}}$, which indicated 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 $\lambda_{max,\mathbf{2a–f}}$, which implied that the Pro1 residue is crucial for the catalytic activities of 4-OT. Third, **1** and **2a** were incubated with synthetic 4-OT[19] and the rate of decrease of absorbance at $\lambda_{max,\mathbf{2a}}$ 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 ^{1}H 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 λ_{max} of **2a–f**, standard workup 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**, whereas **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 analysis 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

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 NaH_2PO_4 buffer (pH 5.5) yielding chiral γ-nitroaldehydes **3a–g**.

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

[a] Isolated yields. [b] Determined by GC or HPLC analysis with chiral stationary phase.[17] [c] Determined by HPLC analysis with chiral stationary phase and/or optical rotation.[17] [d] Compared to nitroolefin. [e] Previous result.[13]

the chiral centers of the major enantiomers of **3a–g**, respectively, all have 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, whereas **3c** has an *R* configuration. The deviant configuration of **3c** is due to different prioritization of the substituents at the chiral center relative 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 nonenzymatic 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 analysis and ^{1}H NMR spectroscopy. In contrast to **2c**, nonenzymatic 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), which demonstrated that formation of **3a–f** is solely the result of 4-OT-catalyzed Michael-type additions and not of nonenzymatic addition of **1** to **2a–f**. In the case of **2c**, nonenzymatic water addition resulted in the formation of 4-methyl-1-nitropentan-2-ol as confirmed by ^{1}H NMR spectroscopy and GC analysis.

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 that are valuable precursors for GABA analogues. Yields up to 74% and *ee* values up to 98% were established, which demonstrated 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 $\leq$2.5 h are generally shorter, than in the scarce conventional organocatalytic methodologies for identical type of reactions.[9,17] Despite a relatively low molecular weight considering enzymes, the molecular weight of 4-OT is still considerably higher than those of organocatalysts[9] that are able to catalyze acetaldehyde addition to nitroolefins. Bearing this in mind, an alternative for defining efficiency on the basis of the applied mol% of catalyst and reaction time is to assess efficiency by the weight amount of product (in terms of milligrams) that is produced per weight amount of used catalyst per unit of reaction time ($mg_{product}\ mg_{catalyst}^{-1}\ h^{-1}$). Applying the latter definition, the 4-OT-based biocatalytic methodology and the most potent organocatalytic[9e] methodology, to the best of our knowledge, are equally efficient for the Michael-type addition of acetaldehyde (**1**) to nitrostyrene (**2g**).[9,17,21] This observation in combination with the broad substrate scope of this 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]

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Keywords: aminobutyric acid · biocatalysis · C–C bond formation · enzymes · Michael-type addition

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[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 (see Scheme S1 in the Supporting Information). 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] Concentrations of **2a–f** were adjusted on the basis of their specific ε_{max} values and solubility properties. See the Supporting Information for details.

[16] Concentration of acetaldehyde and 4-OT was adjusted on basis of concentration of nitroolefin (**2a–f**).

[17] See the Supporting Information for details.

[18] **2a–f** have different λ_{max} values. See the Supporting Information for details.

[19] Synthetic 4-OT was purchased from GenScript USA Inc. (Piscataway, NJ). For folding of chemically synthesized 4-OT into an active homohexamer, see: M. C. Fitzgerald, I. Chernushevich, K. G. Standing, S. B. H. Kent, C. P. Whitman, *J. Am. Chem. Soc.* **1995**, *117*, 11075–11080.

[20] A Williamson ether synthesis of **3b** with monohalogenated cyclopentane (i.e., chloro-, bromo- or iodocyclopentane) gives **3a**.

[21] See reference [9e] for the most efficient organocatalytic methodology, 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 ($mg_{\mathbf{3g}}\ mg_{catalyst}^{-1}\ h^{-1}$): 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 $_{\mathbf{3g}}\ mg_{catalyst}^{-1}\ h^{-1}$). 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 ($0.72\ mg_{\mathbf{3g}} mg_{4-OT}^{-1}\ h^{-1}$).

[22] T. Narancic, J. Radivojevic, P. Jovanovic, D. Francuski, M. Bigovic, V. Maslak, V. Savic, B. Vasiljevic, K. E. O'Connor, J. Nikodinovic-Runic, *Bioresour. Technol.* **2013**, *142*, 462–468.

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