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Stereochemical Control of Enzymatic Carbon-Carbon Bond-Forming Michael-type Additions by 'Substrate Engineering'

Yufeng Miao,[a] Pieter G. Tepper,[a] Edzard M. Geertsema[a,b] and Gerrit J. Poelarends*[a]

[a] Dr. Y. Miao, Mr. P.G. Tepper, Dr. E.M. Geertsema, Prof. Dr. G.J. Poelarends
Department of Chemical and Pharmaceutical Biology
Groningen Research Institute of Pharmacy, University of Groningen
Antonius Deusinglaan 1, 9713 AV Groningen (The Netherlands)
E-mail: g.j.poelarends@rug.nl
Web: http://www.rug.nl/staff/g.j.poelarends/

[b] Present address: Institute for Life Science and Technology, Hanze University of Applied Sciences, Zernikeplein 11, 9747 AS Groningen

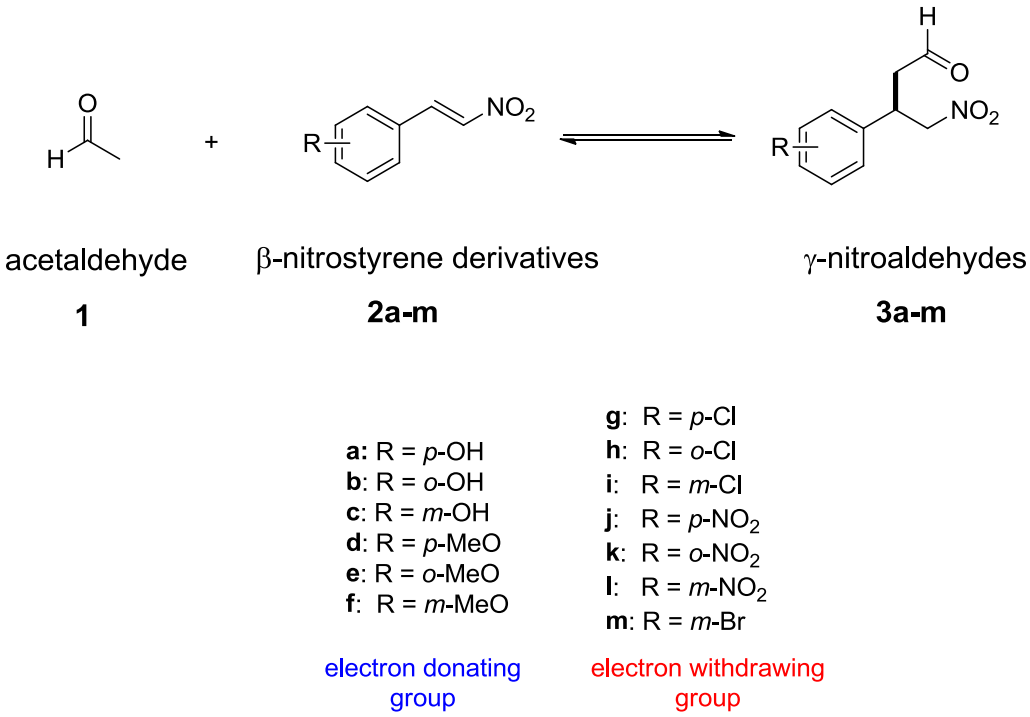
Abstract

The enzyme 4-oxalocrotonate tautomerase (4-OT) promiscuously catalyzes the Michael-type addition of acetaldehyde to β-nitrostyrene derivatives yielding chiral γ-nitroaldehydes, which are important precursors for pharmaceutically active γ-aminobutyric acids. In this study, we investigated the effect of different substituents at the aromatic ring of the Michael acceptor on catalytic efficiency and stereoselectivity of the 4-OT-catalyzed acetaldehyde addition reactions. Highly enantioenriched (*R*)- and (*S*)-γ-nitroaldehydes and 4-substituted chroman-2-ol can be obtained with good to excellent yields by applying different substituents at appropriate positions of the aromatic substrate. The stereochemical control of these enzymatic Michael-type additions by 'substrate engineering' allows enantioselective synthesis of valuable γ-aminobutyric acid precursors. In addition, the results suggest a novel enzymatic synthesis route towards precursors for chromans and derivatives, which are valuable scaffolds for preparing biologically active natural products.

Introduction

γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter widely distributed in the mammalian central nervous system to regulate neuronal excitation.[1] GABA deficiency has been associated with several neurodegenerative disorders such as Parkinson's disease,[2] Huntington's disease[2b, 3] and Alzheimer's disease,[4] and psychiatric disorders including depression,[5] alcoholism[6] and anxiety.[5b-c, 7] Over the past few decades, a number of GABA derivatives showing biological activities have been developed as potential drugs for the treatment of neurological disorders.[8, 9] It has been shown that the biological activities of these compounds are largely dependent on the configuration of the chiral center. For example, only the (*S*)-enantiomer of Vigabatrin,[10] the (*R*)-enantiomer of Baclofen,[11] and the (*S*)-enantiomer of Pregabalin[12] are the active pharmaceutical ingredients. The asymmetric synthesis of GABA derivatives has therefore attracted considerable attention and several methodologies using metal-, organo- and biocatalysts, or their combinations, to construct these chiral molecules have been established.[8a]

We have recently reported a biocatalytic approach for the asymmetric Michael-type addition of acetaldehyde to nitroalkenes to afford enantioenriched γ-nitroaldehydes,[13] which can be readily converted into chiral GABA derivatives by two simple chemical steps. This methodology utilizes the catalytic promiscuity of the enzyme 4-oxalocrotonate tautomerase (4-OT).[14] In this study, we investigated the effect of different substituents at the aromatic ring of the Michael acceptor on catalytic efficiency and stereoselectivity of the 4-OT-catalyzed Michael-type addition reactions. We report the asymmetric 4-OT-catalyzed Michael-type addition of acetaldehyde (**1**, Scheme 1) to a series of mono-substituted β-nitrostyrene derivatives (**2c**-**m**) yielding enantioenriched (*R*)- or (*S*)-γ-nitroaldehydes (**3c**-**m**), as well as the 4-OT-catalyzed Michael-type addition of **1** to *trans*-2-hydroxy-β-nitrostyrene (**2b**), which, after cyclization, affords 4-(nitromethyl)-chroman-2-ol (**5**, Table 1).



Scheme 1. Michael-type addition of acetaldehyde (**1**) to β-nitrostyrene derivatives (**2a-m**)

Results and Discussion

The 4-OT-catalyzed reaction of donor **1** with a series of nitrostyrene derivatives (**2b-l**) as potential acceptors (Scheme 1) was examined. In separate analytical-scale experiments, **2b-l** and **1** were incubated with 4-OT in NaH_2PO_4 buffer containing an appropriate co-solvent (Table S1). The reactions were followed by monitoring the change in absorbance at λ_{max} of **2b-l** by UV-spectroscopy. During all reactions, a decrease in the absorbance of **2b-l** was observed in course of time, which indicated depletion of these nitroolefins (Figure S1-S10). Incubations of **1** with **2b-l** under the same conditions but in the absence of 4-OT showed negligible decreases in absorbances at $\lambda_{max,\ \mathbf{2b\text{-}l}}$, indicating that 4-OT is responsible for catalysis (Figure S1-S10).

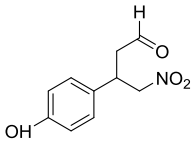
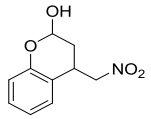
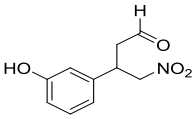
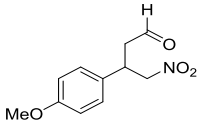
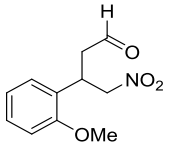
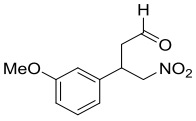
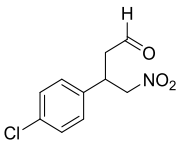
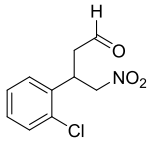
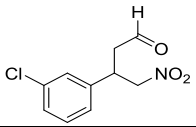
Next, preparative-scale experiments were performed to allow product identification by NMR spectroscopy and hence to confirm that the 4-OT-catalyzed additions of **1** to **2b-l** give Michael-type addition adducts **3b-l**. In addition, the enzymatic preparation of **3m** from **2m**

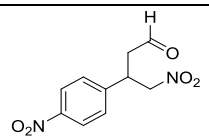
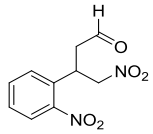
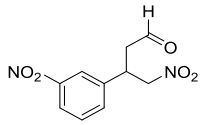
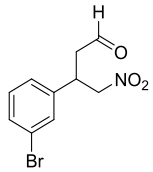
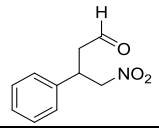
and **1** was investigated. In separate experiments, **2b**-**m**, **1**, and 4-OT were incubated in NaH_2PO_4 buffer with an appropriate co-solvent (Table 1, Table S2). The progress of each reaction was monitored by UV-spectroscopy (Figure S11-S14). After the reaction was completed, work-up and purification procedures were carried out to isolate the enzymatic products. NMR spectroscopic analysis of the purified products confirmed the formation of γ-nitroaldehydes **3c-m** (Figure S16-S25). Intriguingly, the expected γ-nitroaldehyde **3b** (from **1** and **2b**) was not observed. Instead, the formation of 4-(nitromethyl)-chroman-2-ol (**5**) as a result of hemiacetalization of **3b** was established by NMR spectroscopy (Figure S15) and comparison with literature data.[15] The formed 4-substituted chroman-2-ol (**5)** is a synthetically valuable intermediate, which can be easily converted into chromans and their derivatives with antimicrobial and anticancer properties.[16] Good to excellent yields (up to 96%) were achieved for products **3c**-**f**, **3h**-**i**, **3m** and **5** (Table 1). The somewhat lower yields of **3j**-**l** (31-37%) were caused by the low conversion rates with nitrostyrene derivatives **2j-l** and the non-enzymatic hydration of **2j-l** in aqueous reaction media.

The enzymatically obtained γ-nitroaldehydes **3c**-**m** were reduced to the corresponding alcohols **4c**-**m** for the determination of their enantiomeric excesses (*ee*) by chiral phase HPLC. Good to excellent *ee* values (between 82% and 97%) were achieved for **3c**-**m**, indicating that 4-OT is highly stereoselective during the catalytic process (Table 1; Figure S46-S55). The absolute configuration of **3c**-**m** was determined by optical rotation. Interestingly, comparison with literature data revealed that the *meta*- and *para*-substituted γ-nitroaldehydes (**3d**, **3f**, **3g**, **3i**, and **3j**) have the (*S*)-configuration, whereas the *ortho*-substituted γ-nitroaldehydes **3e** and **3k** have the (*R*)-configuration (Table 1, Table S3). The observed negative optical rotation of *meta*-substituted γ-nitroaldehydes **3c**, **3l** and **3m** could not be compared with literature data since, to the best of our knowledge, these compounds have not been reported in the literature so far. We assume the major enantiomers of **3c**, **3l** and **3m** to have the (*S*)-configuration

because they showed the same negative optical rotation and elution order in HPLC chromatograms as observed with the *meta*-substituted products **3f** and **3i**. Similarly, the absolute configuration of the major enantiomer of **3h** is tentatively assigned as *R* by comparing its optical rotation data with those of **3e** and **3k**.

Table 1. Preparative-scale asymmetric 4-OT-catalyzed Michael-type additions of acetaldehyde **1** (50 eq.) to β-nitrostyrene derivatives **2a-n** (1.5-2.0 mM) in NaH_2PO_4 buffer (pH 5.5) yielding γ-nitroaldehydes **3a-n** or chroman-2-ol **5**.

entry	β-Nitro-styrene	Product		*t* [h]	Conversion [%]	Yield[a] [%]	*ee*[b] [%]	Abs. Conf.[c]	Co-solvent (v/v)	4-OT [mol %][d]
1[e]	**2a**		**3a**	0.3	n.d.	65	51	*S*	EtOH 10%	5.6
2[f]	**2b**		**5**	6.1	96	95	n.d.[i] *syn*/*anti:* 1.8/1	n.d.	EtOH 10%	1.0
3	**2c**		**3c**	0.7	98	94	95	*S*	EtOH 10%	1.0
4	**2d**		**3d**	3.0	99	85	94	*S*	DMSO 40%	1.0
5	**2e**		**3e**	1.0	99	93	97	*R*	DMSO 40%	1.0
6	**2f**		**3f**	0.7	92	81	96	*S*	DMSO 40%	1.0
7[g]	**2g**		**3g**	2.5	n.d.	51	69	*S*	DMSO 45%	2.8
8	**2h**		**3h**	2.8	98	94	82	*R*	EtOH 25%	1.0
9	**2i**		**3i**	3.9	93	93	84	*S*	EtOH 25%	1.0

10	**2j**		**3j**	3.3	83	37	87	*S*	DMSO 40%	1.0
11	**2k**		**3k**	3.7	48	31	94	*R*	DMSO 40%	1.0
12	**2l**		**3l**	4.0	84	35	95	*S*	DMSO 40%	1.0
13	**2m**		**3m**	1.2	98	96	92	*S*	DMSO 40%	1.0
14[h]	**2n** (R=H)		**3n**	2.0	n.d.	70	81	*S*	EtOH 10%	1.4

[a] Isolate yield. [b] Determined by HPLC analysis with a chiral stationary phase. [c] Absolute configurations of major enantiomers of **3c**-**m** were determined by optical rotation and comparison with literature data (see Table S3 in the Supporting information). [d] Compared to β-nitrostyrene derivatives **2a**-**n**. [e] Previous results, reaction performed with **1:** 50 mM, **2a**: 1.3 mM, 4-OT: 73 μM in phosphate buffer (pH 7.3).[16a] [f] Diastereomeric ratio (*dr*) was determined by ^{1}H NMR and comparison with literature data;[18] enantiomeric excess of **5** was not determined. [g] Previous results, reaction performed with **1**: 65 mM, **2g**: 1.3 mM, 4-OT: 36 μM in phosphate buffer (pH 5.5).[16c] [h] Previous results, reaction performed with **1**: 50 mM; **2n**: 2 mM; 4-OT: 28 μM in phosphate buffer (pH 5.5).[16b] [i] n.d. = not determined.

Kinetic studies of the 4-OT-catalyzed addition of **1** to **2a**-**o** (**2o**: R = *para*-fluoro) were carried out to elucidate the influence of substituents on the kinetic parameters. To address the influence of co-solvent and pH on kinetic parameters, the kinetic assays of the 4-OT-catalyzed reaction with *trans*-β-nitrostyrene (**2n**) were performed at different pH values and in different solvent systems (Table 2, entry 1-4). We have previously reported that lowering the pH of the reaction media from 7.3 to 5.5 gives an ~4-fold increase in both k_{cat} and K_M, resulting in an unchanged catalytic efficiency (k_{cat}/K_M) (Table 2, entry 1 and 2). Interestingly, switching the co-solvent of the reaction medium from EtOH (10%, v/v) to DMSO (40%, v/v) also resulted in a slight increase (up to 2-fold) in both k_{cat} and K_M (Table 2, entry 3 and 4, compare to entry 1 and 2). The kinetic data obtained with **2a** in two different solvent systems showed similar

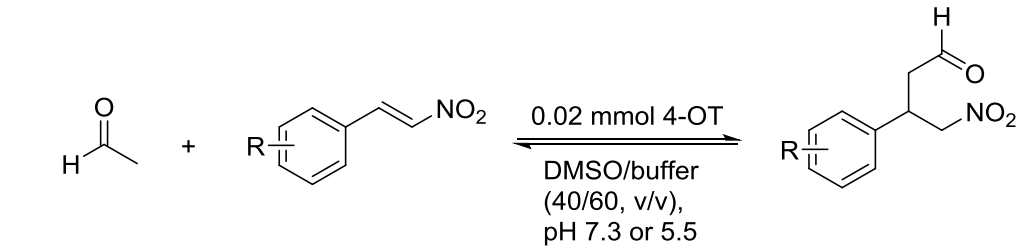
changes in k_{cat} and K_M values (Table 2, entry 5-6), suggesting that this solvent effect on the kinetic parameters is consistent for the different nitroolefins. This effect may be caused by a small conformational change in 4-OT's structure induced by the co-solvent DMSO.[17]

The kinetic parameters of the 4-OT-catalyzed additions of **1** to **2a**-**o** were determined and compared with those of **2n** (R=H) measured under the same solvent and pH conditions (Table 2). In case of *para*-substituted nitrostyrenes, an increase in k_{cat} for substrates carrying electron donating groups (hydroxyl and methoxyl group) and a decrease in k_{cat} for the substrate with a strongly electron withdrawing group (nitro group) were observed (Table 2, compare entry 6-10 to entry 3). The k_{cat} seems not to be significantly affected by fluoro- and chloro-substituents. The obtained K_M values suggest that the affinity of 4-OT for *para*-substituted nitrostyrenes is affected by the hydrophilicity of the substituents. Substituents with high hydrophilicity generally result in an increase in K_M value, indicating a lower affinity of 4-OT towards the substrate (Table 2, entry 6, 8, 9, compare to entry 3). The highest K_M values were obtained with hydroxyl- and fluoro-substituted nitrostyrenes (Table 2, entry 6 and 8).

Results obtained with *meta*-substituted nitrostyrenes show similar electronic effects caused by the substituents. The presence of electron donating groups (hydroxyl and methoxyl group) enhances the k_{cat}, while the presence of a strongly electron withdrawing group (nitro group) shows the opposite effect. Furthermore, the affinity of 4-OT towards *meta*-substituted nitrostyrenes seems to be influenced by the hydrophilicity of the substituents as well, as the bromo- and chloro-substituted nitrostyrenes showed the lowest K_M values (Table 2, entry 13-14, compare to entry 3) while a more hydrophilic substituent (hydroxyl group) at the *meta*-position leads to an increased K_M value (Table 2, entry 11, compare to entry 3). The K_M values obtained with *meta*-substituted nitrostyrenes are generally somewhat lower than those determined for the corresponding *para*-substituted substrates, suggesting that nitrostyrene derivatives with a *meta*-substitution fit better to the activity site of 4-OT. The relatively low

K_M values obtained with *meta*-substituted nitrostyrenes also result in higher k_{cat}/K_M values compared to that obtained with **2n** (Table 2, entry 11-15, compare to entry 3).

Table 2. Apparent kinetic parameters for the 4-OT-catalyzed Michael-type addition of acetaldehyde (**1**) to *para*-, *meta*-, and *ortho*-substituted β-nitrostyrenes (**2a**-**o**)



1 **2a-o** **3a-o**

entry		R	Substrate	k_{cat} (s^{-1}) × 10^3	K_M for **2** (mM)	k_{cat}/K_M (M^{-1} s^{-1})	pH	Co-solvent
1[a]		H	**2n**	17	0.25	68	7.3	EtOH
2[b]	no substitution	H	**2n**	70	1.1	66	5.5	EtOH
3[c]		H	**2n**	33±1	0.46±0.06	68±3	7.3	DMSO
4[d]		H	**2n**	118±12	1.84±0.29	64±6	5.5	DMSO
5		*p*-OH[a]	**2a**	60	1.6	37	7.3	EtOH
6		*p*-OH	**2a**	150±2	4.34±0.80	34±5		DMSO
7	*para*-	*p*-MeO	**2d**	61±2	0.64±0.05	95±3		DMSO
8	substitution	*p*-F	**2o**	39±1	1.35±0.09	28±1		DMSO
9		*p*-Cl	**2g**	33±1	0.69±0.05	47±2		DMSO
10		*p*-NO_2	**2j**	15±1	0.46±0.06	33±2		DMSO
11		*m*-OH	**2c**	71±3	0.67±0.09	106±5	7.3	DMSO
12		*m*-MeO	**2f**	103±6	1.00±0.14	103±6		
13	*meta*- substitution	*m*-Br	**2m**	44±1	0.17±0.02	260±8		
14		*m*-Cl	**2i**	31±8	0.19±0.02	168±5		
15		*m*-NO_2	**2l**	24±1	0.34±0.05	71±3		
16		*o*-OH	**2b**	31±1	0.67±0.04	46±1	5.5[e]	DMSO
17	*ortho*-	*o*-MeO	**2e**	270±22	4.75±0.54	57±5		
18	substitution	*o*-Cl	**2h**	174±8	0.92±0.09	187±9		
19		*o*-NO_2	**2k**	139±27	4.54±1.19	31±6		

[a] Previously reported data, assays were performed in EtOH/phosphate buffer (10/90, v/v) at pH 7.3.[16a] [b] Previously reported data, assays were performed in EtOH/phosphate buffer (10/90, v/v) at pH 5.5.[16c] [c] Assays were performed in DMSO/phosphate buffer (40/60, v/v) at pH 7.3. [d] Assays were performed in DMSO/phosphate buffer (40/60, v/v) at pH 5.5. [e] Assays were performed in DMSO/phosphate buffer (40/60, v/v) at pH 5.5 to prevent nonenzymatic hydration of nitrostyrene derivatives **2b**, **2e**, **2h** and **2k** in the aqueous solvent system.

The results of the kinetic assays with *ortho*-substituted substrates show that introducing a methoxyl group to the *ortho*-position of nitrostyrene significantly increases the value of k_{cat}, while the presence of a hydroxyl group at this position results in a significant decrease in k_{cat} (Table 2, entries 16 and 17, compare to entry 4). The different K_M values observed for the different *ortho*-substituted nitrostyrenes may be related to steric effects.

Conclusion

4-OT catalyzes the asymmetric Michael-type addition of acetaldehyde to various *ortho*-, *meta*-, and *para*-substituted β-nitrostyrenes. For most of the γ-nitroaldehydes excellent yields (up to 96%) and high optical purities (*ee* values up to 97%) were achieved. Enzymatic access to (*R*)-γ-nitroaldehydes was achieved with *ortho*-substituted nitrostyrenes, while (*S*)-γ-nitroaldehydes were obtained with *para*- and *meta*-substituted nitrostyrenes. Apparently, attaching substituents to the *ortho*-position of the aromatic substrate induces steric effects, causing either substrate re-positioning in the active site of 4-OT or a stereofacial shielding effect. Introducing electron donating groups at the *meta*- and *para*-position of nitrostyrene derivatives could significantly enhance the catalytic rates (up to 5-fold improvement in k_{cat}). Together with the ongoing enzyme engineering studies in our group, this 'substrate engineering' work is an important step towards our aim of developing novel "Michaelases" for carbon-carbon bond-forming Michael-type addition reactions with high efficiency and stereoselectivity. Finally, the 4-OT-catalyzed reaction between **1** and **2b** does not give γ-nitroaldehyde **3b** as final product; instead, it yields 4-nitromethyl-chroman-2-ol (**5**) as a result of hemiacetalization between the aldehyde and hydroxyl groups of presumed intermediate **3b**. This Michael-type addition-cyclization cascade reaction provides exciting options for the enzymatic synthesis of precursors for chromans and derivatives, which are valuable scaffolds for preparing biologically active natural products.

Experimental Section

For experimental procedures and characterization of compounds, see Supporting Information.

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Keywords

Catalytic promiscuity; Michael-type additions; substrate engineering; γ-nitroaldehydes; γ-aminobutyric acids

Table of Contents Entry

Key Topic: Enzyme Promiscuity

Short text: The enzyme 4-oxalocrotonate tautomerase promiscuously catalyzes the Michael-type addition of acetaldehyde to β-nitrostyrene derivatives. Highly enantioenriched (*R*)- or (*S*)-γ-nitroaldehydes and 4-substituted chroman-2-ol can be obtained with good to excellent yields by applying different substituents at appropriate positions of the aromatic substrate.

TOC graphic: