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Communication

Enzyme Promiscuity

Stereochemical Control of Enzymatic Carbon–Carbon Bond-Forming Michael-Type Additions by "Substrate Engineering"

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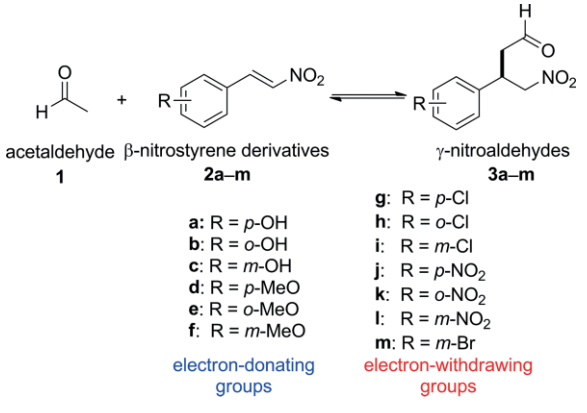
Abstract: The enzyme 4-oxalocrotonate tautomerase (4-OT) promiscuously catalyzes the Michael-type addition of acetaldehyde to β-nitrostyrene derivatives to yield chiral γ-nitroaldehydes, which are important precursors for pharmaceutically active γ-aminobutyric acids. In this study, we investigated the effect of different substituents at the aromatic ring of the Michael acceptor on the 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 could be obtained in good to excellent yields by applying different substituents at appropriate positions of the aromatic substrate. Stereochemical control of these enzymatic Michael-type additions by "substrate engineering" allowed the 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] in addition to psychiatric disorders including depression,[5] alcoholism,[6] and anxiety.[5b,5c,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 involving the use of metal catalysts, organocatalysts, and biocatalysts, or their combinations, to construct these chiral molecules have been established.[8a]

We 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 effects of different substituents at the aromatic ring of the Michael acceptor on the catalytic efficiency and stereoselectivity of 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 monosubstituted β-nitrostyrene derivatives (see compounds **2c**–**m**) to yield enantioenriched (*R*)- or (*S*)-γ-nitroaldehydes (see compounds **3c**–**m**), as well as the 4-OT-catalyzed Michael-type addition of **1** to (*E*)-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**.

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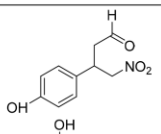
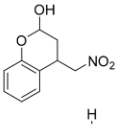
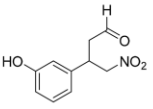
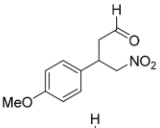
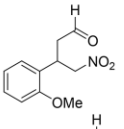
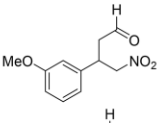
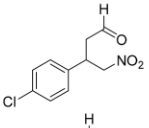
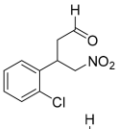
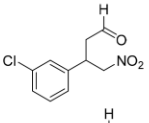
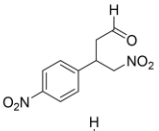
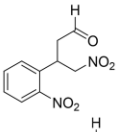
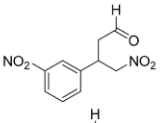
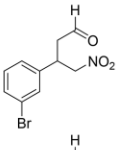
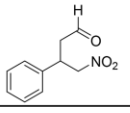
Table 1. Preparative-scale asymmetric 4-OT-catalyzed Michael-type additions of acetaldehyde **1** (50 equiv.) to β-nitrostyrene derivatives **2a**–**n** (1.5–2.0 mM) in NaH_2PO_4 buffer (pH 5.5) to yield γ-nitroaldehydes **3a**–**n** or chroman-2-ol **5**.

Entry	β-Nitrostyrene	Product		*t* [h]	Conversion [%]	Yield[a] [%]	*ee*[b] [%]	Absolute configuration[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.[g] *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[h]	**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[i]	**2n** (R = H)		**3n**	2.0	n.d.	70	81	(*S*)	EtOH 10%	1.4

[a] Yield of isolated product. [b] Determined by HPLC analysis with a chiral stationary phase. [c] Absolute configurations of the major enantiomers of **3c**–**m** were determined by optical rotation measurements and comparison with literature data (see Table S3). [d] Compared to β-nitrostyrene derivatives **2a**–**n**. [e] Previously reported result; reaction performed with **1** (50 mM), **2a** (1.3 mM), 4-OT (73 μM) in phosphate buffer (pH 7.3).[13a] [f] Diastereomeric ratio (*dr*) was determined by ^{1}H NMR spectroscopy and comparison with literature data;[15] enantiomeric excess (*ee*) of **5** was not determined. [g] n.d. = not determined. [h] Previously reported result; reaction performed with **1** (65 mM), **2g** (1.3 mM), 4-OT (36 μM) in phosphate buffer (pH 5.5).[13c] [i] Previously reported result; reaction performed with **1** (50 mM), **2n** (2 mM), 4-OT (28 μM) in phosphate buffer (pH 5.5).[13b]

Results and Discussion

The 4-OT-catalyzed reaction of donor **1** with 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 in the Supporting Information). The reactions were monitored by recording the change in the absorbance at λ_{max} of **2b**–**l** by UV spectroscopy. During all reactions, a decrease in the absorbance of **2b**–**l** was observed over the course of time, which indicated depletion of these nitro olefins (Figures S1–S10). Incubation of **1** with **2b**–**l** under the same conditions but in the absence of 4-OT showed a negligible decrease in the absorbance at λ_{max} of **2b**–**l**, which indicated that 4-OT was responsible for the catalysis (Figures 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** gave 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 (Figures S11–S14). Upon completion of the reaction, workup and purification procedures were performed to isolate the enzymatic products. Analysis of the purified products by NMR spectroscopy confirmed the formation of γ-nitroaldehydes **3c**–**m** (Figures 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] 4-Substituted chroman-2-ol **5** is a synthetically valuable intermediate that 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 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.

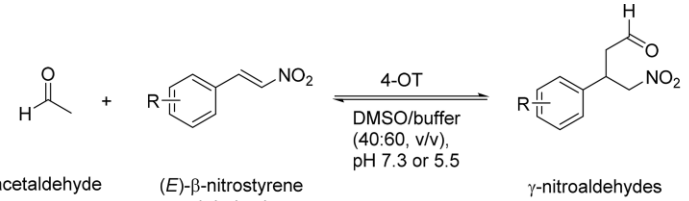
Enzymatically obtained γ-nitroaldehydes **3c**–**m** were reduced to the corresponding alcohols **4c**–**m** for determination of their enantiomeric excess (*ee*) values by chiral stationary phase HPLC. Good to excellent *ee* values (between 82 and 97 %) were achieved for **3c**–**m**, which indicated that 4-OT was highly stereoselective during the catalytic process (Table 1; Figures S46–S55). The absolute configurations of **3c**–**m** were determined by optical rotation measurements. Interestingly, comparison with literature data revealed that the *meta*- and *para*-substituted γ-nitroaldehydes (i.e., compounds **3d**, **3f**, **3g**, **3i**, and **3j**) had the (*S*) configuration, whereas *ortho*-substituted γ-nitroaldehydes **3e** and **3k** had 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, as, to the best of our knowledge, these compounds have so far not been reported. We assume that the major enantiomers of **3c**, **3l**, and **3m** have the (*S*) configuration, because they showed the same negative optical rotation and elution order in the HPLC chromatograms as those observed with *meta*-substituted products **3f** and **3i**. Similarly, the absolute configuration of the major enantiomer of **3h** was tentatively assigned as (*R*) by comparing its optical rotation data with those of **3e** and **3k**.

Kinetic studies of the 4-OT-catalyzed addition of **1** to **2a**–**o** (**2o**: R = *para*-fluoro) were performed to elucidate the influence of substituents on the kinetic parameters. To address the influence of the co-solvent and pH on the kinetic parameters, the kinetic assays of the 4-OT-catalyzed reaction with (*E*)-β-nitrostyrene (**2n**) were performed at different pH values and in different solvent systems (Table 2, Entries 1–4). We previously reported that lowering of the pH of the reaction media from 7.3 to 5.5 gave an approximately fourfold increase in both the turnover rate (k_{cat}) and Michaelis constant (K_M), which resulted in an unchanged catalytic efficiency (k_{cat}/K_M) (Table 2, Entries 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 twofold) in both k_{cat} and K_M (Table 2, compare Entries 3 and 4 with Entries 1 and 2). The kinetic data obtained with **2a** in two different solvent systems showed similar changes in the k_{cat} and K_M values (Table 2, Entries 5 and 6), which suggests that this solvent effect on the kinetic parameters is consistent for the different nitro olefins. This effect may be caused by a small conformational change in the structure of 4-OT induced by the DMSO co-solvent.[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 the case of *para*-substituted nitrostyrenes, an increase in k_{cat} for substrates carrying electron-donating groups (e.g., hydroxy and methoxy groups) and a decrease in k_{cat} for the substrate with a strongly electron-withdrawing group (e.g., nitro group) were observed (Table 2, compare Entries 6–10 with Entry 3). The k_{cat} value seemed 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 resulted in increased K_M values, which indicates a lower affinity of 4-OT towards the substrate (Table 2, compare Entries 6, 8, and 9 with Entry 3). The highest K_M values were obtained with hydroxy- and fluoro-substituted nitrostyrenes (Table 2, Entries 6 and 8).

Results obtained with *meta*-substituted nitrostyrenes showed similar electronic effects caused by the substituents. The presence of electron-donating groups (e.g., hydroxy and methoxy groups) enhanced the k_{cat} values, whereas the presence of a strongly electron-withdrawing group (e.g., nitro group) showed the opposite effect. Furthermore, the affinity of 4-OT towards *meta*-substituted nitrostyrenes seemed 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, compare Entries 13 and 14 with Entry 3), whereas a more hydrophilic substituent (e.g., hydroxy group) at the *meta* position led to an increased K_M value (Table 2, compare Entry 11 with Entry 3). The K_M values obtained with *meta*-substituted nitrostyrenes were generally

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**.



acetaldehyde **1** | (*E*)-β-nitrostyrene and derivatives **2a–o** | γ-nitroaldehydes **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	H	**2n**	70	1.1	66	5.5	EtOH
3[c]	substitution	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		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	7.3	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		
12		*m*-MeO	**2f**	103±6	1.00±0.14	103±6		
13	*meta*	*m*-Br	**2m**	44±1	0.17±0.02	260±8	7.3	DMSO
14	substitution	*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		
17	*ortho*	*o*-MeO	**2e**	270±22	4.75±0.54	57±5	5.5[e]	DMSO
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.[13a] [b] Previously reported data; assays were performed in EtOH/phosphate buffer (10:90, v/v) at pH 5.5.[13c] [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.

somewhat lower than those determined for the corresponding *para*-substituted substrates, which suggests that nitrostyrene derivatives with a *meta* substitution fit better to the active site of 4-OT. The relatively low K_M values obtained with *meta*-substituted nitrostyrenes also resulted in higher k_{cat}/K_M values than that obtained with **2n** (Table 2, compare Entries 11–15 with Entry 3).

The results of the kinetic assays with *ortho*-substituted substrates show that introducing a methoxy group into the *ortho* position of nitrostyrene significantly increased the value of k_{cat}, whereas the presence of a hydroxy group at this position resulted in a significant decrease in k_{cat} (Table 2, compare Entries 16 and 17 to Entry 4). The different K_M values observed for the different *ortho*-substituted nitrostyrenes may be related to steric effects.

Conclusion

4-OT was shown to catalyze 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, whereas (*S*)-γ-nitroaldehydes were obtained with *para*- and *meta*-substituted nitrostyrenes. Apparently, attaching substituents to the *ortho* position of the aromatic substrate induced steric effects, which caused either substrate repositioning in the active site of 4-OT or a stereofacial shielding effect. Introducing electron-donating groups at the *meta* and *para* positions of the nitrostyrene derivatives significantly enhanced the catalytic rates (up to fivefold 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** did not give γ-nitroaldehyde **3b** as the final product; instead, it yielded 4-(nitromethyl)chroman-2-ol (**5**) as a result of hemiacetalization between the aldehyde and hydroxy 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.

Supporting Information (see footnote on the first page of this article): Experimental procedures and characterization of the compounds.

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Keywords: Catalytic promiscuity · Enzyme catalysis · Michael addition · Substituent effects · Substrate engineering

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