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RESEARCH ARTICLE

Stereodivergent Biocatalytic Formal Reduction of α-Angelica Lactone to (*R*)- and (*S*)-γ-Valerolactone in a One-Pot Cascade

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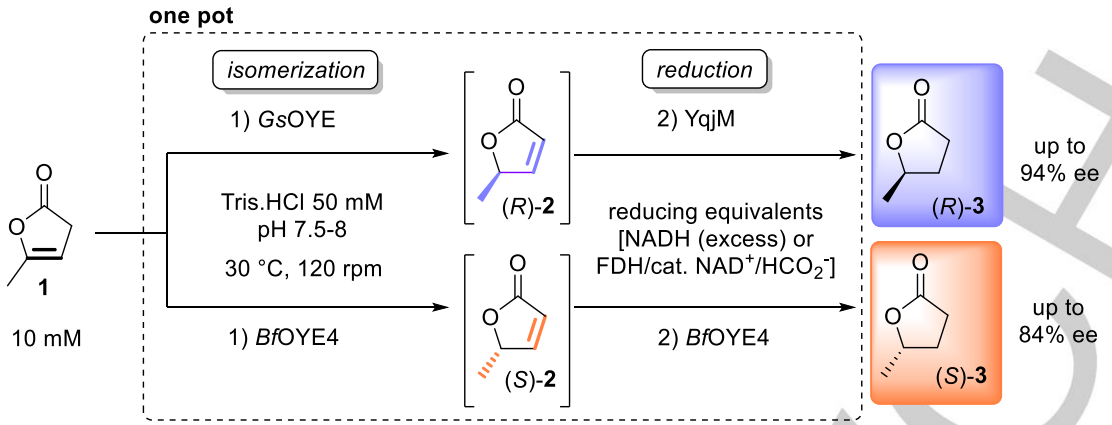
Abstract: The formal asymmetric and stereodivergent enzymatic reduction of α-angelica lactone to both enantiomers of γ-valerolactone was achieved in a one-pot cascade by uniting the promiscuous stereoselective isomerization activity of Old Yellow Enzymes with their native reductase activity. In addition to running the cascade with one enzyme for each catalytic step, a bifunctional isomerase-reductase biocatalyst was designed by fusing two Old Yellow Enzymes, thereby generating an unprecedented case of an artificial enzyme catalyzing the reduction of nonactivated C=C bonds to access (*R*)-valerolactone in overall 41% conversion and up to 91% ee. The enzyme *Bf*OYE4 could be used as single biocatalyst for both steps and delivered (*S*)-valerolactone in up to 84% ee and 41% overall conversion. The reducing equivalents were provided by a nicotinamide recycling system based on formate and formate dehydrogenase, added in a second step. This enzymatic system provides an asymmetric route to valuable chiral building blocks from an abundant bio-based chemical.

Introduction

Chiral synthons are nowadays highly demanded by pharma and chemical industries.[1] Angelica lactones and γ-valerolactone are versatile bio-based building blocks derived from glucose and represent platform chemicals with broad industrial applications, such as solvent production, fuel additives, precursors of bio-based polymers, building blocks of biologically active molecules, fragrance and flavoring agents.[2] Most of the chemical methods available for the isomerization of prochiral α-angelica lactone to chiral β-angelica lactone and subsequent reduction to chiral γ-valerolactone require strong acid or base catalysts producing racemic products in low yields.[2a, 3] A simplified procedure described the use of iron as catalyst and ethanol in excess for the base-free transfer hydrogenation of α-angelica lactone at 100 °C.[4] Direct access to enantiomers of γ-valerolactone from α-angelica lactone in a stereoselective manner has been rarely investigated. Asymmetric hydrogenation was possible employing a BINAP-Ru(II) complex, however the (*S*)-enantiomer of γ-valerolactone was obtained with poor enantiopurity (20% ee).[5] To the best of our knowledge, no protocol has reported the use of enzymes for this reaction in one step. In general, the asymmetric reduction of nonactivated C=C bonds by enzymes, such as in the case of α-angelica lactone, has been poorly exploited for synthetic applications. A unique case of stereoselective reduction of isolated (unfunctionalized) C=C bonds was reported, in which a FAD-dependent geranylgeranyl reductase could catalyze the reduction of farnesol derivatives with very high stereoselectivity (ee up to >99%) under nitrogen atmosphere in a diluted substrate solution (1 mM).[6]

We reported in 2016 a nonselective enzymatic protocol toward racemic γ-valerolactone from α-angelica lactone (**1**) with the Old Yellow Enzyme OYE2 from *Saccharomyces cerevisiae*[7] that proceeded through a formal reduction of nonactivated alkene over two steps, however with low titers (max. 2 mM of racemic product).[8] The first step of isomerization proceeded non stereoselectivity to the formation of the intermediate β-angelica lactone (**2**), which was reduced in the second step by the same enzyme to furnish racemic γ-valerolactone (**3**). Following our recent work on the asymmetric isomerization of α-angelica lactone and the identification of stereocomplementary Old Yellow Enzymes (OYEs) to access both enantiomers of β-angelica lactone,[9] we considered the design of a cascade for the asymmetric formation of (*R*)- and (*S*)-γ-valerolactone (Scheme 1), thus providing a straightforward and stereoselective route to both enantiomers of this important chiral building block. The nonnatural activity of OYEs in the isomerization reaction is redox neutral and acid-base catalyzed,[9] while the second step relies on the native nicotinamide-dependent reducing activity of these flavoproteins on activated alkenes.[10] In the latter reaction, the reducing



Scheme 1. Enzymatic cascade for the formal reduction of α-angelica lactone (**1**) into the two enantiomers of γ-valerolactone (**3**) without isolation of the intermediate β-angelica lactone (**2**). Other OYEs tested in the reductive step include OYE2, NCR, and *Gs*OYE, see text for details.

equivalents can be provided either directly by external addition of NAD(P)H, or through the use of a nicotinamide recycling system that utilizes a hydride source, such as glucose or formate, and a dehydrogenase, such as glucose dehydrogenase (GDH) or formate dehydrogenase (FDH), respectively, in combination with catalytic amounts of NAD(P)$^+$.[11]

Results and Discussion

Isomerization and reduction as individual reactions

The cascade combining the nicotinamide-independent asymmetric isomerization of **1** with the nicotinamide-dependent reduction of **2** would ideally imply in the first step the catalytic activity of one OYE with high isomerase activity and stereoselectivity, and in the second step, the catalytic activity of one OYE with high reductase activity. If both enzymes were to be present at the onset of the reaction, no competition with deleterious effect should be observed, such as the reductase showing isomerase activity with poor stereoselectivity, as this would impact the ee of the final product **3**.

We recently identified two highly stereoselective isomerases toward both enantiomers of **2**:[9] *Gs*OYE from *Galdieria sulphuraria* showed (*R*)-enantioselectivity in the isomerization of **1**, while *Bf*OYE4 from *Botryotinia fuckeliana* provided stereocomplementary access to (*S*)-**2** with exquisite ee values. Given the targeted strategy to couple the asymmetric isomerization of **1** to the subsequent reduction of **2** by OYEs, several enzymes were tested for the reduction of *rac*-**2** according to the native nicotinamide-dependent activity of OYEs,[10a] in order to evaluate their activity and enantioselectivity. In the cascade context and the case of the reduction of a pure enantiomer of **2** to **3**, the implementation of an enzyme that is enantioselective for the corresponding intermediate (*R*)- or (*S*)-**2** is preferred, noting that a nonselective enzyme such as OYE2[8] with sufficient reducing activity could still be employed. However, given the reversibility of the isomerization,[9] the use of a reductase such as OYE2 with strong isomerase activity should be carefully evaluated, as the reaction of the intermediate **2** back to **1** may compete with its reduction, impacting the final product yields.

OYE2, *Bf*OYE4, *Gs*OYE, NCR[12] and YqjM[13] were thus applied to the reduction of *rac*-**2** in the presence of catalytic amounts of NAD$^+$ and a nicotinamide recycling system based on ammonium formate and a commercially available formate dehydrogenase (FDH). Except for nonselective OYE2 and NCR, the enzymes were found (*R*)-enantioselective in the reduction of *rac*-**2**, with various levels of activity and low to moderate enantioselectivity (E-value from 3 to 12, Table 1). The E-value obtained by computing the ee values of substrate **2** and product **3**[14] is only apparent since the isomerization is reversible, leading to formation of **1**, which is unstable and hydrolyzes,[9] and is responsible for incomplete recovery (see Figure S1, Supporting Information). The values still allow to discriminate between highly and poorly selective systems, especially in the light of the ee values of both **2** and **3**. Additionally, the same panel of enzymes was tested in the isomerization of **1**. The data indicate that the enzymatic isomerization activity is highly enzyme-dependent and is uncoupled from the respective reductase activity. YqjM was poorly active while NCR showed low level of nonselective activity (Table 2).

Table 1. Reduction of 10 mM *rac*-**2** to **3** by purified OYE homologues.[a]

Entry	Enzyme	[**3**] (mM)	Ee of **3** (%)	Ee of **2** (%)	E-value (apparent)[d]
1	Control (no enzyme)	n.d.	n.a.	*Rac.*	n.a.
2	OYE2	7.1	*Rac.*	n.a.[e]	<1
3	*Bf*OYE4	6.4	13 (*R*)	>99 (*S*)	5
4	*Gs*OYE	2.2	36 (*R*)	23 (*S*)	3
5	YqjM[b]	3.8	67 (*R*)	77 (*S*)	12
6	NCR[c]	7.9	6 (*R*)	n.a.[e]	n.a.

[a] Reaction conditions: 10 mM *rac*-**2**, 2 vol% DMSO, ~5 μM of enzyme (200 μg/mL), Tris.HCl buffer (50 mM, pH 7.5), 0.05 eq. NAD$^+$, 2 eq. NH_4HCO_2, FDH (commercial, 5 mg/mL), 30 °C, overnight. [b] 20 μM FMN was added to enhance flavination.[13, 15] [c] 1.5 eq. of NADH in place of the recycling system was used. [d] Tool for calculation of E-value (enantioselectivity)[14] available in open access at http://biocatalysis.uni-graz.at/biocatalysis-tools/enantio. [e] **2** was fully consumed. n.a. not applicable; n.d. not detected.

Table 2. Relative activity of a panel of purified OYEs in the isomerization of **1**.[a]

Entry	Enzyme	Relative activity[b] (%)	Ee of **2** (%)
1	OYE2	100	*Rac*
2	*Gs*OYE	70	79 (*R*)
3	*Bf*OYE4	24	87 (*S*)
4	YqjM	2	11 (*S*)
5	NCR	15	3 (*S*)

[a] Reaction conditions: 10 mM **1**, 2 vol% DMSO, ~5 µM of enzyme (200 µg/mL), Tris.HCl buffer (50 mM, pH 7.5), 5 h, 30 °C. [b] Relative activity with OYE2 activity set as 100%, based on amount of product **2** formed.

One-pot cascade for the reduction of 1 to 3

The isomerization of **1** to **2** was combined with the subsequent reduction of **2** to access **3** in a one-pot cascade. The first strategy relied on a concurrent cascade using a single enzyme and was initially tested with both stereocomplementary *Gs*OYE and *Bf*OYE4, and the nonselective enzyme OYE2, given that these enzymes, in addition to good activity as isomerase, also demonstrated moderate to excellent activity as reductase on **2** (see Tables 1 and 2). However, such a design, in which the enzyme (10 µM), the substrate **1** and the cofactor in excess (1.5 eq. NADH) were present from the onset of the reaction, led to very low recovery after 6 h of reaction time (~30%), mostly as a mixture of **1** with traces of **2** and **3** (data not shown). Further efforts to trigger conversion to **3** remained unsuccessful, and despite several control experiments, we could not identify the reason for this behavior, but suspected the degradation of **1** as major competing reaction. In addition, reduction of FMN by NADH can take place following the native activity of OYE, and may favor O_2 reduction[17] in a competing reaction to the isomerization, thereby letting **1** incubate in the reaction mixture, which are conditions favorable for spontaneous hydrolysis over time (see Figure S1, Supporting Information).[9] We could demonstrate that after a shorter reaction time (30 min) at pH 9 in absence of NADH, isomerization by *Gs*OYE took place and produced (*R*)-**2**, but no conversion was observed in presence of 1.5 eq. of NADH, under otherwise identical reaction conditions (data not shown).

A sequential strategy was therefore devised, in which the cofactor was supplied after the isomerization had taken place. In addition, to prevent the reverse isomerization of **2** as a competing reaction to the reduction, the enzyme used as isomerase was deactivated by a 10 min incubation at 90 °C. After cool down, the reduction was then initiated by addition of the reductase and the reducing equivalents. Overall, this design provides a one-pot two-step bi-enzymatic access to **3**. The general set-up was first tested by combining OYE2 as the most active isomerase with either *Bf*OYE4, *Gs*OYE, NCR, OYE2, OYE3, or YqjM, as reductase. Isomerization was run for 3 h at 30 °C, followed by deactivation of OYE2 at 90 °C, and further incubation overnight at 30 °C after addition of NADH and the reductase. The system was active in the cascade (see Table S1, Supporting Information) and provided compound **3** in varying amounts (from 1.8 to 7.2 mM) and ee values (from *rac*. to 69% ee for (*R*)-**3**), along with traces of **2** (no traces of **1** detected).

With the aim to generate **3** in high enantiopurity, the isomerase was changed to (*R*)-stereoselective *Gs*OYE to access (*R*)-**3**, and to (*S*)-stereoselective *Bf*OYE4 to access (*S*)-**3**. In addition to the standard protocol, further adjustments to boost the conversion and favorably impact the ee of the product were implemented, including longer isomerization time and switching from an excess of NADH to a nicotinamide FDH-based recycling system (catalytic amounts of NAD^+/FDH/2 eq. of ammonium formate). The selection of a suitable enzyme pair isomerase/reductase appeared crucial to reach highest products titers and enantiomeric excess (Table 3). Firstly, the issue arising from using a reductase with strong isomerase activity was highlighted in the combination of the isomerase *Bf*OYE4 with OYE2. The two-step cascade relying on excess of NADH applied to 10 mM of **1** delivered only 0.7 mM of **3**, likely due to reverse isomerization from **2** to **1** by OYE2 and further hydrolysis (see Entry 1, Table 3). Therefore, the enzymes YqjM and NCR showing poor isomerase activity but moderate to strong reductase activity were chosen for the second step of the cascade (see Tables 1-2). In the (*R*)-selective cascade, the highest ee values obtained (92-93%) were however accompanied by the lowest product titers (2.6-3.1 mM, Entries 6 and 9, Table 3). The best compromise between enantiopurity and product titer was obtained by applying *Gs*OYE in the isomerization overnight, followed by reduction by YqjM employed as cell-free extract in combination with the nicotinamide recycling system (4.3 mM of (*R*)-**3** were produced with 84% ee, Entry 8, Table 3). The formation of (*S*)-**3** reached 2.1 mM in up to 75% ee, by combining the *Bf*OYE4-catalyzed isomerization at pH 8 for 5 h with the reduction by NCR in presence of the recycling system. Following the isomerization, a short incubation at pH 9 was implemented, promoting the degradation of residual **1**,[9] with the aim to prevent competing nonselective isomerization by NCR. This contributed to enhanced ee value and product titer (see Entries 4-5, Table 3).

Table 3. Bi-enzymatic sequential cascade on 10 mM of **1** with stereoselective isomerase and selected paired reductase.[a]

Entry	Isomerase	Reductase	NADH source	[**3**] (mM)	Ee of **3** (%)
1	*Bf*OYE4[b]	OYE2	1.5 eq.	0.7	13 (*S*)
2	*Bf*OYE4[b]	YqjM	1.5 eq.	2.3	64 (*S*)
3	*Bf*OYE4[b]	YqjM	recycling system	1.7	58 (*S*)
4	*Bf*OYE4[b]	NCR	recycling system	1.9	62 (*S*)
5	*Bf*OYE4[b,c]	NCR	recycling system	2.1	75 (*S*)
6	*Gs*OYE	YqjM	1.5 eq.	2.6	93 (*R*)
7	*Gs*OYE[d]	YqjM	recycling system	4.4	77 (*R*)
8	*Gs*OYE[d]	YqjM[e]	recycling system	4.3	84 (*R*)

[a] Reaction conditions: 1) Isomerization: 10 mM of **1**, 2 vol% DMSO, ~5 µM of isomerase (200 µg/mL), Tris.HCl buffer (50 mM, pH 7.5), 30 °C, 3 h; 2) Denaturation: 10 min at 90 °C followed by cool down to 30 °C; 3) Reduction: ~5 µM of reductase (200 µg/mL), NADH source as indicated (either 1.5 eq. NADH or formate (2 eq.)/FDH (10 U/mL)/NAD^+(0.05 eq.)), 30 °C, overnight. [b] pH 8. [c] Isomerization 5 h, then pH adjustment to 9 and incubation for 30 min, then back to 7.5 for denaturation/reduction. [d] Isomerization overnight. [e] YqjM as cell-free extract preparation.

Design of a bi-functional fusion protein

The direct reduction via formal *trans*-hydrogenation by OYEs operates exclusively on C=C bonds that are activated by an electron withdrawing group, which lowers the electron density and favors attack of the hydride onto Cβ.[10a] The absence of a conjugated activating group explains for instance the high regioselectivity on polyunsatured compounds,[11, 17] in which only 1,4-addition can take place. By fusing two Old Yellow Enzymes with high isomerase and high reductase activity respectively, a bi-functional protein can be obtained that bypasses the absence of reactivity of nonactivated olefins toward reduction, such as in the case of α-angelica lactone. Some cases of bi-functional biocatalyst preparations involving OYEs have been reported. They rely on co-immobilization of OYEs with a nicotinamide recycling enzyme such as GDH,[18] or the fusion of an OYE and a monooxygenase, as shown with the OYE XenB and a Baeyer-Villiger monooxygenase.[19] Fusion proteins with dual catalytic activity involving two OYEs are to the best of our knowledge not known. We opted for fusing *Gs*OYE with YqjM, based on previous data (see Table 3), in order to obtain an (*R*)-reductase active on **1**. As demonstrated in several cases of cascade reactions, the use of fusion enzymes may confer additional advantage connected to improved catalytic efficiency due to substrate channeling.[20]

Given the importance of the C-terminus for active site architecture of YqjM and substrate recognition,[21] the linker was designed as a nine amino acid stretch (SGGSGGSGG) joining the N-terminus of YqjM and the C-terminus of *Gs*OYE. The protein *Gs*9*Bs*-OYE was further expressed with a N-terminus His-tag, resulting in a protein of 83.6 kDa that showed good soluble expression in *E. coli* (see Figure S2, Supporting Information). The protein was obtained after purification via immobilized metal affinity chromatography on a HisTrap FF column, followed by two size-exclusion chromatography steps on a HiLoad 16/600 Superdex 200 and Superdex 75, respectively, in order to remove all possible contaminations (see Figures S2-S4, Supporting Information). Both isomerase and reductase activities of the fusion protein were tested separately. The isomerization of **1** to **2** was studied at different pHs (7.5, 8 and 9) for 3 h. Since YqjM was shown to have poor isomerization activity with a slight preference toward formation of (*S*)-**2** (see Table 2), the results obtained with the fusion protein can be mostly attributed to *Gs*OYE (see Table 4). The data indicate that the fusion protein conserved the (*R*)-stereoselectivity displayed by *Gs*OYE as single component isomerase and was most active at pH 8, achieving 1620 TTN (8.1 mM, Entry 2, Table 4, Figure S8, Supporting Information).

In order to analyze the reductive activity of the fusion protein, 2-methyl-2-cyclopenten-1-one (**4**) and dimethyl itaconate (**5**) were chosen as substrates, in addition to **2** (see Table S2, Supporting Information). The reduction of **4** provides hints on the reductase activity of both components. The data obtained after incubation overnight in presence of the FDH-recycling system indicate that the reductive activity of *Gs*OYE and YqjM on **4** is additive in the fusion protein. In contrast, **5** is only accepted by YqjM.[22] Investigating the reduction of **5** can therefore provide information on the activity of YqjM within the fusion protein. A slight improvement in the reduction of **5** by YqjM was observed when employed as fusion protein (see Entry 3, Table S2). Lastly, the reduction of *rac*-**2** performed with *Gs*9*Bs*-OYE confirms that the fusion protein is active for the targeted purpose and, as observed with the individual enzymes (see Table 1), is (*R*)-selective. After 3 h reaction, the product titers barely increased and the ee of the final product dropped, confirming the moderate enantioselectivity in the kinetic resolution of *rac*-**2** (see Figure S5, Supporting Information). In general, it can be concluded that fusing the two proteins did not alter their functional activity.

Table 4. Isomerization of 10 mM of **1** to (*R*)-**2** using the fusion protein *Gs*9*Bs*-OYE at different pHs.[a]

Entry	pH	[**2**] (mM)	Ee of **2** (%)
1	7.5	4	75 (*R*)
2	8	8.1	74 (*R*)
3	9	6.3	51 (*R*)

[a] Reaction conditions: 10 mM **1**, 2 vol% DMSO, ~5 µM of enzyme (400 µg/mL), Tris.HCl buffer (50 mM, pH 7.5), 3 h, 30 °C.

Formal asymmetric reduction of 1 to (*R*)-3 catalyzed by the bi-functional fusion protein

The asymmetric reduction of **1** to (*R*)-**3** by *Gs*9*Bs*-OYE was performed in Tris.HCl buffer (50 mM, pH 7.5 or 8) in a two-step fashion, as this was found crucial to obtain the final product (*vide supra*). The isomerization was first run for 3 h at 30 °C at 120 rpm. The reducing equivalents were then supplemented in form of catalytic amounts of NAD^+, 2 eq. of ammonium formate and FDH, and the reaction was run overnight at 30 °C. The concentrations of *Gs*9*Bs*-OYE and FDH, as well as the pH and reaction time of the isomerization, were varied in order to identify the optimum conditions. In general, it could be observed that higher product titers of (*R*)-**3**, especially when higher enzyme concentrations were employed (see Entries 1-2 and 9-11, Table 5), correlated with lower ee values, as was also observed in the bi-enzymatic cascade (see Table 3). This is in line with a moderately efficient kinetic resolution happening at the stage of the reduction of the nonenantiopure intermediate **2**. The ee of (*R*)-**3** were surprisingly higher than the values obtained in the cascade conducted with the two individual enzymes, highlighting the advantage of using the bi-functional protein. Ee value of 91% and good product titer (4.1 mM) were obtained with 0.048 mol% of catalyst (4.8 µM, 400 µg/mL, see Entry 6, Table 5). It is noteworthy that the amount of intermediate product **2** varied from case to case (see Entries 6 and 7, Table 5). The use of lyophilized whole cells of *E. coli* expressing *Gs*9*Bs*-OYE (50 mg/mL) was found compatible with the reaction (Entry 12, Table 5, Figure S9, Supporting Information), and data comparable with those obtained with the use of the purified protein were obtained.

Finally, following the isomerization step performed for 5 h with 4.8 µM of purified *Gs*9*Bs*-OYE, the whole reaction mixture was diluted by adding the components of the recycling system and additional buffer. This furnished 2.9 mM (*R*)-**3** in 93% ee, and thus the highest conversion to (*R*)-**3** of 58%, considering the dilution factor of two.

Table 5. Reduction of 10 mM of **1** to (*R*)-**3** by the bi-functional protein *Gs*9*Bs*-OYE.[a]

Entry	[*Gs*9*Bs*-OYE] (µM)	[FDH] (mg/mL)	[**2**] (mM)	Ee of **2** (%)	[**3**] (mM)	Ee of **3** (%)
1	1.2	5	2.5	34 (*R*)	1.6	>99 (*R*)
2	2.4	5	1.7	11 (*S*)	3.4	91 (*R*)
3	3.6	5	1.5	25 (*S*)	3.9	90 (*R*)
4	4.8	10	1.6	21 (*S*)	4.1	91 (*R*)
5	4.8	20	2.6	25 (*S*)	2.8	94 (*R*)
6	4.8[b,c]	5	3.3	12 (*R*)	4.1	91 (*R*)
7	4.8[b,d]	5	1.7	17 (*S*)	4.2	90 (*R*)
8	4.8	5	1.4	44 (*S*)	4.8	89 (*R*)
9	7.1	5	1.1	67 (*S*)	5.1	86 (*R*)
10	9.5	5	1.0	67 (*S*)	5.5	83 (*R*)

[a] Reaction conditions: 10 mM **1**, 2 vol% DMSO, *Gs*9*Bs*-OYE (as indicated), Tris.HCl buffer (50 mM, pH 8), 3 h, 30 °C, 120 rpm. Then, 0.05 eq. NAD^+, 2 eq. NH_4HCO_2, FDH (as indicated), 30 °C, 120 rpm, overnight. [b] pH 7.5. [c] Isomerization overnight. [d] Isomerization for 5 h. [e] 50 mg/mL lyophilized whole cells.

Monoenzymatic two-step one-pot cascade to (*S*)-3 with *Bf*OYE4

Finally, to address the low yield in the formation of (*S*)-**3** (see Table 3), a simplified system relying on *Bf*OYE4 as isomerase and reductase was investigated. The reaction was conducted at pH 8, as it favors the isomerization by *Bf*OYE4,[9] and with increased enzyme concentration (~10 µM, 400 µg/mL). After 5 h reaction time at 30 °C, the components of the FDH-based recycling system were added and incubation was continued overnight at 30 °C. After the first step, formation of 6.1 mM of (*S*)-**2** could be monitored with 96% ee, and after the second step, (*S*)-**3** was obtained in 4.1 mM and 84% ee, without detectable amounts of **1** and only traces of (S)-**2** (see Figure S7, Supporting Information). This loss can be attributed here again to the reverse isomerization of **2** and the competing hydrolysis occurring on **1** at pH 8.[9] Nevertheless, this set-up provided the best performing system to access (*S*)-**3** with the highest enantiopurity and good titers.

Conclusion

While several methods have reported access to enantiomers of γ-valerolactone from levulinic acid, complementary methods from α-angelica lactone are rare. The enzymatic protocol reported here provides an alternative route that can be easily modulated to access both enantiomers of γ-valerolactone on demand in up to 94% ee. Product titers are satisfactory and current efforts are targeting further reaction engineering approaches to reach higher yields. To access (*R*)-γ-valerolactone, we constructed an artificial bi-functional enzyme by fusing *Gs*OYE and YqjM, two Old Yellow Enzymes with complementary isomerase and reductase activity, respectively. (*S*)-γ-valerolactone was obtained with *Bf*OYE4, which fulfilled both catalytic roles. Crucial was to operate the cascade in two steps, by providing the reducing equivalents for the second reduction reaction after the isomerization step. These results provide encouraging perspectives for the design of enzymatic one-pot cascades for the formal reduction of nonactivated alkenes.

Experimental Section

Enzymes

*Gs*OYE,[9] *Bf*OYE4,[1] OYE2,[14] YqjM[23] and NCR[23] were produced according to published procedures. Formate dehydrogenase was from Evocatal.

Biotransformations

All biotransformations were performed in duplicates.

*Typical procedure for the isomerization reaction of **1** to **2***

An aliquot of enzyme (200 or 400 µg/mL) was added to a solution containing the substrate added from a 500 mM stock solution in DMSO (final concentration 10 mM) in Tris.HCl buffer (50 mM, pH as indicated, 500 µL final volume). The samples were incubated at 30 °C and 120 rpm for the indicated time and then extracted with ethyl acetate (2 x 250 µL) spiked with 1-decanol (0.1 vol%) as internal standard. The combined organic phases were dried on anhydrous Na_2SO_4 and analyzed on chiral-phase GC for both conversion and optical purity.

Typical procedure for the reduction reaction

An aliquot of enzyme (200 or 400 µg/mL) was added to a solution containing the substrate added from a 500 mM stock solution in DMSO (final concentration 10 mM), the cofactor NADH added from a 250 mM stock solution in Tris.HCl buffer (final concentration 15 mM), and was completed to 500 µL with Tris.HCl buffer (50 mM, pH as indicated). The samples were incubated at 30 °C and 120 rpm for the indicated time and analysis was performed by GC after extraction as described for the isomerization reaction.

In the case of the use of the nicotinamide recycling system, NADH was replaced with 0.5 mM NAD^+ added from a 100 mM stock solution in buffer, 20 mM of NH_4HCO_2 added from a 1 M stock solution in buffer and FDH (commercial, 5 mg/mL, added from a 200 mg/mL stock solution in buffer).

Typical procedure for the one-pot cascade reaction with the fusion protein Gs9Bs-OYE or BfOYE4

An aliquot of enzyme (400 µg/mL) was added to a solution containing the substrate added from a 500 mM stock solution in DMSO (final concentration 10 mM) in Tris.HCl buffer (50 mM, pH as indicated, 500 µL final volume). The samples were incubated at 30 °C and 120 rpm for the indicated time. Then 0.5 mM NAD^+ from a 100 mM stock solution in buffer, 20 mM of NH_4HCO_2 from a 1 M stock solution in buffer and FDH (commercial, 5 mg/mL, or as indicated, added from a 200 mg/mL stock solution in buffer) were added to the reaction mixture and incubation was pursed at 30 °C for the indicated time. Analysis was performed by GC after extraction as described for the isomerization reaction.

Details on the preparation of the fusion protein and the analytical methods can be found in the supporting information.

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Keywords: Asymmetric reduction • Biocatalysis • Isomerization • Lactone • Old Yellow Enzyme

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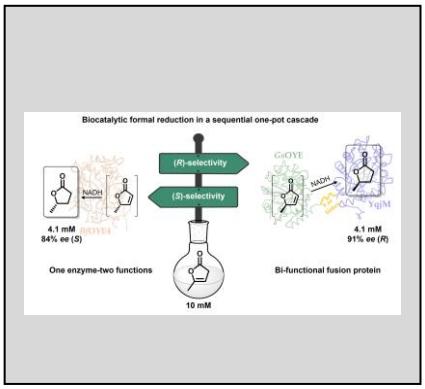

The enzymatic formal reduction of nonactivated α-angelica lactone to both enantiomers of γ-valerolactone was achieved in a biocatalytic one-pot cascade combining an asymmetric isomerization and a reduction. A fusion protein linking two Old Yellow Enzymes with complementary activities showed excellent (*R*)-selectivity, while the (*S*)-enantiomer was obtained using a single Old Yellow Enzyme for both steps of the cascade.

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