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Nicotinamide Adenine Dinucleotide-Dependent Redox-Neutral Convergent Cascade for Lactonizations with Type II Flavin-Containing Monooxygenase

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Abstract. A nicotinamide adenine dinucleotide (NADH)-dependent redox-neutral convergent cascade composed of a recently discovered type II flavin-containing monooxygenase (FMO-E) and horse liver alcohol dehydrogenase (HLADH) has been established. Two model reaction cascades were analyzed for the synthesis of *γ*-butyrolactone and chiral bicyclic lactones. In the former cascade, all substrates were converted into one single *γ*-butyrolactone with high atom efficiency. More than 130 mM *γ*-butyrolactone were obtained when applying 100 mM cyclobutanone and 50 mM 1,4-butanediol in this cascade. In the second cascade where bicyclo[4.2.0]octan-7-one and cyclohexanedimethanol were coupled, the ketone substrate was converted to the corresponding normal lactone with an *ee* value of 89–74% (3a*S*, 7a*S*) by FMO-E alone and the abnormal lactone with an *ee* value of >99% (3a*R*, 7a*S*) was formed by both HLADH and FMO-E.

Keywords: Baeyer-Villiger Monooxygenase; Redox-neutral Cascade; Cofactor Specificity; Alcohol Dehydrogenase; Biocatalysis

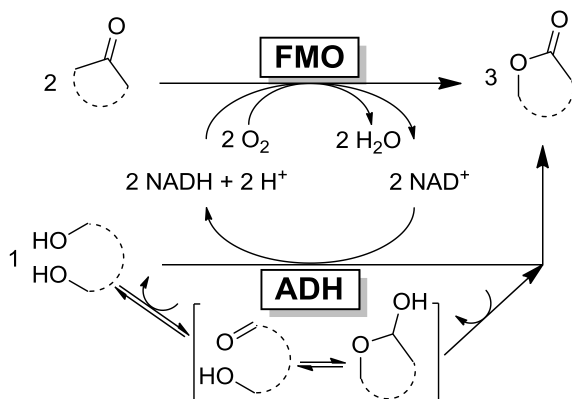
Nature uses an elegant synthetic strategy by building multi-step biotransformations *via* coupling of enzymes. By doing so, complex molecules are synthesized from simple structures, toxic or unstable intermediates are converted *in situ*, and reversible reactions are driven to completion.[1] The elegance and efficiency of natural cascades and networks also explains the increasing popularity of domino- or cascade reactions in organic synthesis in general. Especially in redox biocatalysis, cascade reactions are attractive since with the so-called redox-neutral cascades (also referred as "self-sufficient" or "closed loop") expensive cofactors can be recycled *in situ* without an additional cofactor regeneration system.[2]

Recently, our group reported a concept of a redox-neutral convergent cascade reaction consisting of an alcohol dehydrogenase (ADH) and a cyclohexanone monooxygenase (CHMO).[3] The cofactor regeneration of this cascade system is achieved *via* oxidation of the "double-smart cosubstrate" 1,6-hexanediol catalyzed by the ADH. Two molar equivalents of cyclohexanone and one molar equivalent of 1,6-hexanediol were converted into a single product *ε*-caprolactone with high atom efficiency. However, this cascade is currently limited to NADPH due to the strict cofactor recognition of the CHMO from *Acinetobacter* sp. NCIMB 9871 ($CHMO_{Acineto}$),[4] a well-known Baeyer-Villiger monooxygenase (BVMO) for lactone synthesis. From an industrial perspective, NADH is the preferred cofactor as it is cheaper (up to 30 times) and more stable than NADPH.[5] Besides that, it is well documented that it is easier to perform the recycling of NAD^+ than that of $NADP^+$.[6]

The majority of BVMOs including $CHMO_{Acineto}$ belongs to the NADPH-dependent type I BVMOs.[4] Up to date, there are several studies devoted to change the cofactor specificity of BVMOs through protein engineering.[7] One recent study on switching the cofactor specificity of $CHMO_{Acineto}$ has been reported by the group of Bornscheuer.[7e] With the aid of structure analysis, sequence alignments and literature data, they designed variants with three or four mutations exhibiting enhanced activity ratios (=NADH/NADPH) up to 4,200-fold. One $CHMO_{Acineto}$ variant with three mutations (3M variant; S186P_S208E_K326H) showed 10-fold increase in the catalytic efficiency (k_{cat}/K_M) compared to that of

wild type $CHMO_{Acineto}$ towards NADH. It has been shown that 83% of the activity of the wild type CHMO towards NADH results from the uncoupling reaction yielding H_2O_2; on the contrary, the designed CHMO 3M variant exhibits uncoupling activity of only 15%, which demonstrated the power of their protein engineering approach.[7e] Nevertheless, catalytic efficiency (k_{cat}/K_M) of the CHMO 3M variant towards NADPH was 670-fold lower than that of wild type CHMO[7e]. This endorses the fact that designing a CHMO variant for NADH-coupled reactions with a catalytic efficiency is not trivial.

Recently, some of us have identified a new class of flavoprotein monooxygenases, type II flavin-containing monooxygenases (FMOs).[8] Three type II FMOs from *Rhodococcus jostii* RHA1, namely FMO-E, FMO-F, and FMO-G, were used as effective biocatalysts for Baeyer-Villiger oxidations. The most promising feature of these FMOs is that they accept both NADPH and NADH. FMO-E, one of these type II FMOs, could be purified in good yield without losing the flavin adenine dinucleotide (FAD) cofactor. Being fascinated with the extraordinary feature of the FMO-E, we focus on the application of this enzyme in a redox-neutral convergent cascade by coupling it with a NADH-dependent ADH for lactonizations (Scheme 1).



Scheme 1. Convergent cascade reactions coupling the flavin-containing monooxygenase (FMO) with an alcohol dehydrogenase (ADH) for lactonizations. Two molar equivalents of ketone are coupled with one equivalent of diol to synthesize three molar equivalents of lactone.

Our characterization study for pH profile revealed that the FMO-E showed the highest activity at pH 7.5 but it is most stable at pH 6.5 (Figure S3). FMO-E was rather a thermally unstable enzyme as its optimal temperature is only 25 °C and it became inactivated at temperatures above 30 °C (Figure S4). Recently, it has been systematically evaluated that addition of cofactors and coenzymes could improve the enzyme's stability (Goncalves *et al.*, submitted, unpublished results). Increased stability of 4-hydroxyacetophenone monooxygenase – a BVMO – upon coenzyme binding was previously documented by van den Heuvel *et al.* [9] Inspired by those findings, some cofactors, such as flavin adenine dinucleotide (FAD), NADH and NADPH were studied for their effect on the long-term stability of the FMO-E at 30 °C. It was found that the half-life time ($t_{1/2}$) of the FMO-E with 10 μM FAD and 0.1 mM NADPH at 30 °C was 2.6 times longer than without these cofactors (Table 1, Figure S5). On the other hand, NADH did not show any beneficial effect on the stability.

Table 1. Half-life times of FMO-E at 30 °C with different cofactors. Experiments were performed in duplicates.

Cofactor	k_d a) [h^{-1}]	$t_{1/2}$ b) [30 °C, h]	Stabilisation factor [-]
No additive	0.313 ± 0.020	2.21 ± 0.14	1.0
10 μM FAD	0.218 ± 0.004	3.17 ± 0.06	1.5
10 μM FAD + 0.1 mM NADH	0.220 ± 0.022	3.17 ± 0.32	1.5
10 μM FAD + 0.1 mM NADPH	0.119 ± 0.001	5.82 ± 0.04	2.6
10 μM FAD + 2.5 mM NADH	0.309 ± 0.002	2.25 ± 0.02	1.0

a) k_d [h^{-1}] = Deactivation constant.
b) $t_{1/2}$ [h^{-1}] = Half-life time.

Next, we evaluated the steady-state kinetic parameters with a model substrate bicyclo[3.2.0]hept-2-en-6-one (**1a**) using either NADH or NADPH. As previously documented,[8a] FMO-E accepted both nicotinamide cofactors and showed typical Michaelis-Menten saturation kinetics based on the Michaelis-Menten double-substrate equation (Equation S1). FMO-E shows higher affinity towards NADPH (K_M = 3 μM) than NADH (K_M = 10 μM) (Table 2, Figure S6). Even though the k_{cat} for NADH is slightly lower than that for NADPH (2.0 s^{-1} vs 2.8 s^{-1}), FMO-E was shown to be an efficient biocatalyst with NADH as cofactor. The affinity (K_M value) towards the substrate **1a** was found as 2.8 mM (±1.3 mM) using NADPH, and 2.4 mM (±0.7 mM) using NADPH. The k_{cat} value for NADH is about two orders of magnitude higher than that of another reported type II FMO from *Stenotrophomonas maltophilia* (0.029 s^{-1})[10] and it is in the same range of 1–20 s^{-1} with many other class B flavoprotein monooxygenases.[11]

Table 2. Steady-state kinetic parameters of FMO-E for the model substrate bicyclo[3.2.0]hept-2-en-6-one (**1a**) with NADH or NADPH based on the Michaelis-Menten double-substrate equation. Experiments were performed in duplicates.

Cofactor	$K_{M_NAD(P)H}$ [μM]	V_{max} [U/mg]	k_{cat} [s^{-1}]	k_{cat}/K_M [$mM^{-1}s^{-1}$]
NADPH	3 ± 1	2.6 ± 0.4	2.8	1.0
NADH	10 ± 4	1.9 ± 0.2	2.0	0.8

Next, a range of ketone substrates was analyzed with FMO-E using NADH as cofactor (Figure 1). FMO-E showed Baeyer-Villiger oxidation activity towards cyclobutanones **1a**-**1b** and *racemic* fused cyclobutanone **1c**, but no activity was detected with cyclopentanones **1d**-**1e** and cyclohexanone **1f,** partly also demonstrated by Riebel *et al.*[8a] This indicates that the substrate scope of FMO-E is mainly restricted to cyclobutanones. This feature is similar to another type II FMO from *S. maltophilia* as it only converts the standard BVMO substrate **1a** with NADH as cofactor.[10]

The kinetic parameters of FMO-E towards oxidation of cyclobutanone (**1b**) or bicyclo[4.2.0]octan-7-one (**1c**) were determined using NADH as cofactor based on Michaelis-Menten single-substrate equation (Equation S2) by using the NADH concentration fixed at 0.1 mM, which is 10 times higher than the K_M value determined (Table 2). The K_M and k_{cat} values towards substrates **1b** (2.1 mM and 1.3 s^{-1}) and **1c** (1.0 mM and 1.7 s^{-1}) were found to be in the same range as the model substrate **1a** (Figure S7, Table 3). These data also confirm the substrate specificity of FMO-E towards cyclobutanones.

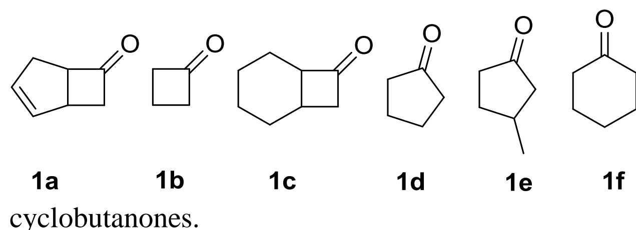


Figure 1. Ketone substrates investigated for FMO-E.

Table 3. Kinetic constants of FMO-E towards cyclobutanone (**1b**) and bicyclo[4.2.0]octan-7-one (**1c**) using NADH as a cofactor (0.1 mM). Experiments were performed in duplicates.

Substrate	K_{M_Sub} [mM]	V_{max} [U/mg]	k_{cat} [s^{-1}]	k_{cat}/K_M [$mM^{-1}s^{-1}$]
1b	2.1 ± 0.2	1.2 ± 0.02	1.3	0.5
1c	1.0 ± 0.1	1.6 ± 0.02	1.7	1.7

Horse liver alcohol dehydrogenase (HLADH) is a widely studied zinc-dependent ADH.[12] It has been applied for decades to synthesize a broad range of lactones, such as *γ*-, *δ*- or *ε*-lactones from diols.[13] Remarkably, HLADH has been used to promote redox biocatalysis using 1,4-butanediol as a "smart cosubstrate", as the thermodynamically stable and kinetically inert coproduct lactone makes the regeneration reaction irreversible.[14] It is worth mentioning here that 1,4-butanediol can be obtained from renewable feedstocks at commercial scale which has been developed by Genomatica Inc. (San Diego, USA)[15] and their fermentative synthesis process has been recently licensed by BASF SE (Germany). HLADH – a well-known ADH for diol oxidation – was chosen to perform the convergent cascade with FMO-E.

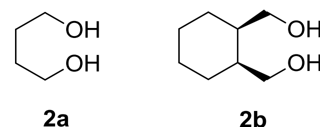
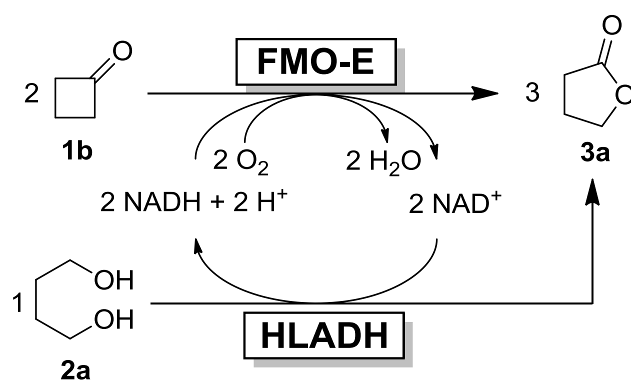


Figure 2. Diol substrates investigated for HLADH.

Two corresponding diols, 1,4-butanediol (**2a**) and cis-1,2-cyclohexanedimethanol (**2b**), were selected as substrates of HLADH (Figure 2) based on the substrate specificity of FMO-E. HLADH showed similar K_M values towards the two diol substrates **2a** and **2b** in the range of 3–10 mM and high K_i values (Figure S8, Table S3) which were calculated based on Michaelis-Menten double-substrate equation with excess substrate inhibition (Equation S3).



Scheme 2. NADH-dependent convergent cascade employing FMO-E and HLADH with cyclobutanone (**1b**) and 1,4-butanediol (**2a**) as substrates to synthesize *γ*-butyrolactone (**3a**).

We selected cyclobutanone (**1b**) (substrate of FMO-E) and the corresponding diol 1,4-butanediol (**2a**) (substrate of HLADH) for the proof-of-concept study of the NADH-dependent convergent cascade (Scheme 2).

Firstly, one positive reaction and four negative controls by eliminating one or two components were carried out with 20 mM substrate (**1b**) and 10 mM 1,4-butanediol (**2a**) (Figure 3). It was shown that the concentration of the lactone product (**3a**) increased to almost 25 mM (analytical yield of 83%) after 24 h and no substrate **1b** could be detected at that time point. However, there were small amounts of 1,4-butanediol left even after 72 h. The reason can be attributed to the evaporation of the ketone substrate **1b** leading to a decreased formation of NAD^+. Therefore, the cofactor regeneration (consequently the whole cascade) became inefficient. Here, it is worth to mention that the reaction was performed in a 30 mL reaction vessel (1 mL total reaction volume) which might be the main reason for the poor mass balance observed in the experiments reported above.

The concentration of 1,4-butanediol increased at 72 h, which is attributed partly to an analytical error. On the other hand, the decrease in the concentration of the lactone after 24 h (Figure 3) can be a result of the hydrolysis of γ-butyrolactone (**3a**) to the corresponding acid, as previously documented in the literature,[3b, 6a, 13d] but also analytical errors should not be neglected. While incubating 20 mM of γ-butyrolactone in 100 mM Tris-HCl buffer at pH 8.0, the rate of autohydrolysis of γ-butyrolactone was found to be 90 µM/h (the rate is doubled at pH 9.0). It is worth to mention here that the half-life time of FMO-E at 20°C without additives was determined as 45 h (data not shown), which is 20 times higher than the half-life time at 30°C (Table 1). In addition, no cyclobutanol – from the ADH-catalyzed reduction of cyclobutanone – was detected as proven by using a standard with GC analytics. The absence of the activity of HLADH towards cyclobutanone (10 mM) reduction was also proven by UV analysis.

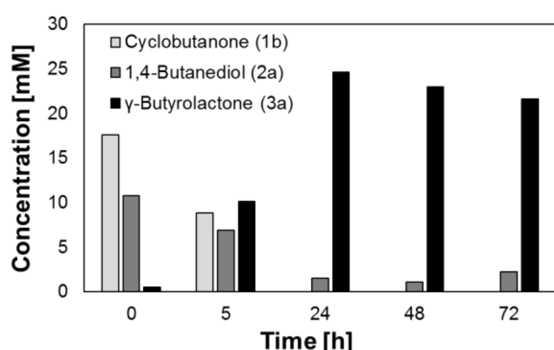


Figure 3. NADH-dependent cascade employing FMO-E and HLADH. Reaction conditions: c(cyclobutanone, **1b**) = 20 mM, c(1,4-butanediol, **2a**) = 10 mM, c(NAD^+) = 1 mM, c(FMO-E) = 1 U (16.6 µM), c(HLADH) = 1 U (7.3 µM), buffer: Tris-HCl (100 mM, pH 8.0), 180 rpm, T = 20°C. Reactions (1 mL in total) run in 30 mL glass-vial.

No target product could be detected in all the negative controls except the one without FMO-E (Figure S9) since the present HLADH can use the oxidized cofactor NAD^+ to synthesize the lactone product.

The reaction system showed approx. 18% depletion in mass balance after 72 h (Figure 3), which to a large extent can be resulted from evaporation of the substrate **1b** and the hydrolysis of the lactone. On the other hand, the lactonization of 1,4-butanediol to γ-butyrolactone consists of three subsequent steps: oxidation of the diol to the hydroxy aldehyde, spontaneous cyclization of the hydroxy aldehyde to lactol and finally oxidation of the lactol to the lactone (Scheme 1). It can be the case that not all diol is converted to lactone, but some amounts are remained as intermediates, which could not be quantified by the GC analytics used in this study.

With the successful proof-of-concept, we further increased the substrates' concentrations to 100:50 mM (ketone:diol). Figure 4A shows that both substrates (**2a**, **1b**) were still unreacted after 72 h using one unit of each enzyme. 1 U/mL (1.1 mg/mL) FMO-E corresponds to 16.6 µM, whereas 1 U/mL (0.3 mg/mL) HLADH corresponds to 7.3 µM based on the standard activity assays (Supporting Information). Turnover number (TON) of the enzymes ($mol_{lactone}/mol_{FMO\text{-}E\ and\ HLADH}$) increased from 904 to 5163, while using 100:50 mM (ketone:diol) instead of 20:10 mM. When two units of each enzyme were used, no ketone substrate could be detected after 48 h (Figure 4B) and the analytical yield of the lactone was achieved as 89% (Table 4). However, the TON value decreased to 2811. In order to evaluate the deactivation of the enzyme while using different enzyme amounts we compared the lactone formation (mM) versus time × total enzyme concentration (h×U mL^{-1}). Both graphs showed a similar progress which eliminates a significant enzyme deactivation by using low amounts of enzymes at least up to the first 5 hours. However, at 24 hours the curve with 2 U/mL of each enzyme (4 U/mL total enzymes) proceeded to higher productivity (Figure S10).

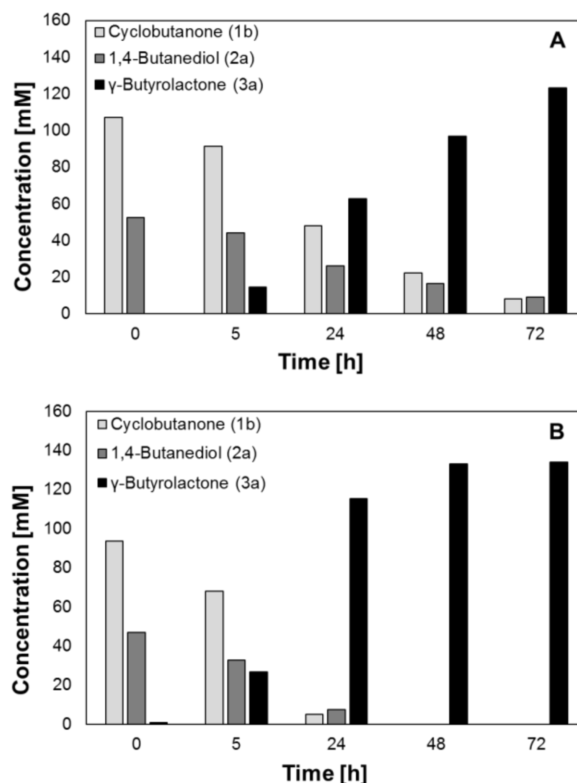


Figure 4. Results of the convergent cascade with high concentration of substrates in 30 mL reactor. Reaction conditions: c(cyclobutanone, **1b**) = 100 mM, c(1,4-butanediol, **2a**) = 50 mM, c(NAD^+) = 1 mM, c(FMO-E) = 1 U (16.6 µM) (**A**), 2 U (33.2 µM) (**B**), c(HLADH) = 1 U (7.3 µM) (**A**), 2 U (14.6 µM) (**B**), buffer: Tris-HCl (100 mM, pH 8.0), 180 rpm, T = 20 °C. Reactions (1 mL in total) run in 30 mL glass-vials.

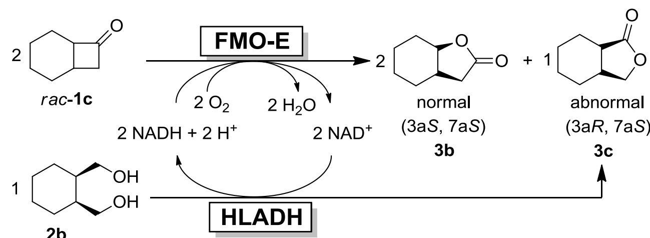
A reactor with a smaller size (1.5 mL) was also used to perform the cascade reaction. A smaller reaction vessel led to decreased evaporation and resulted in full conversion of both substrates (Figure S11A and B). On the other hand, our results indicated that the larger reactor was more suitable when high substrate concentrations were applied (Figure S11C and D). Due to more available molecular oxygen a higher productivity was achieved in the large reactor, (third substrate of the monooxygenation besides the ketone and the cofactor) since the overhead ratio (V_g/V_l) increases from 0.5 (in the small reactor) to 29 (in the large reactor). The $a_{g/l}$ / $V_{reactor}$ (surface area / reactor volume) in case of small reactor was calculated as 133 mm^2/mL and 220 mm^2/mL for the large reactor, whereby in both cases 1 mL of total reaction volume was used. However, more detailed analysis for an accurate determination of the molecular oxygen concentration during the course of the reaction is desirable.

Table 4. Summary of the convergent cascade reactions applied for the synthesis of *γ*-butyrolactone (**3a**).

Reaction number	**1b** [mM]	**2a** [mM]	FMO-E [U/mL] (µM)	HLADH [U/mL] (µM)	5h *c*(**3a**) [mM]	24h *c*(**3a**) [mM]	48h *c*(**3a**) [mM]	72h *c*(GBL) [mM]	Yield [%]	TON[a)] [-]
1	20	10	1.0 (16.6)	1.0 (7.3)	10.1	24.6	23.0	21.6	72	904
2	100	50	1.0 (16.6)	1.0 (7.3)	14.5	62.7	97.0	123.4	82	5163
3	100	50	2.0 (33.2)	2.0 (14.6)	28.7	123.3	133.2	134.1	89	2811

[a)] The TON values represent µmol lactone product formed per total µmol of FMO-E and HLADH.

In the next set of experiments, *racemic* bicyclobutanone substrate bicyclo[4.2.0]octan-7-one (*rac*-**1c**) and the corresponding diol cis-1,2-cyclohexanedimethanol (**2b**) were applied in the convergent cascade system (Scheme 3). According to the work of Riebel *et al.*,[8a] the ratio of the normal (N, **3b**) to abnormal (ABN, **3c**) lactone (of substrate **1c**) catalyzed by FMO-E is determined as 95:5 (N:ABN). Therein, FMO-E was not applied with an *in situ* cofactor regeranation system. Riebel *et al.*,[8a] reported that at 81% of conversion of *rac*-**1c**, the *ee* value of the normal lactone was found as 39% (3a*S*,7a*S*) and the *ee* value of the abnormal lactone was determined as 98% (3a*R*,7a*S*).



Scheme 3. NADH-dependent cascade employing FMO-E and HLADH with racemic bicyclo[4.2.0]octan-7-one (*rac*-**1c**) and cis-1,2-cyclohexanedimethanol (**2b**) as substrates.

In the convergent cascade reaction involving FMO-E and HLADH, we observed the ratio of normal to abnormal lactone decreasing from 3.3:1 (N:ABN, 5 h) to 1.4:1 (N:ABN, 72 h) starting from 20 mM ketone *rac*-**1c** and 10 mM diol substrate **2b** (Figure 5). The diol substrate **2b** was completely converted by HLADH in 48 h, whereas 10 mM ketone substrate *rac*-**1c** were left after 72 h of reaction. The *ee* value of the abnormal lactone **3c** was found to be >99% (*3*a*R*,*7*a*S*), which is the same with the configuration of the product of HLADH (>99%; *3*a*R*,*7*a*S*) reported in the literature.[16] Hence, it was a proof that the FMO-E and HLADH catalyzed the synthesis of the same enantiomer of the abnormal lactone (**3c**). The *ee* value of the normal lactone **3b** was found to be 89% (3a*S*,7a*S*) after 5 h, which however decreased to 74% at 72 h. Overall, the analytical yield of the lactone synthesis was 65% after 72 h of reaction (Figure 5).

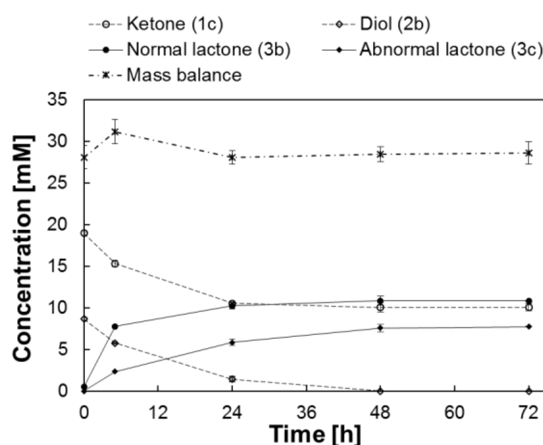


Figure 5. Conversion of bicyclo[4.2.0]octan-7-one (○, *rac*-**1c**) cis-1,2-cyclohexanedimethanol (◇, **2b**) to normal lactone (●, **3b**) and to abnormal lactone (◆, **3c**). Reaction conditions: *c*(bicyclo[4.2.0]octan-7-one, *rac*-**1c**) = 20 mM, *c*(cis-1,2-cyclohexanedimethanol, **2b**) = 10 mM, *c*(NAD^+) = 1 mM, *c*(FMO-E) = 1.0 U (16.6 µM), *c*(HLADH) = 1.0 U (7.3 µM), buffer: Tris-HCl (100 mM, pH 8.0), 180 rpm, T = 20 °C. Reactions (1 mL in total) run in 1.5 mL glass-vials. Standard deviations = 1–7% (experiments performed in duplicates).

For our curiosity, we run the convergent cascade reaction with bicyclo[4.2.0]octan-7-one (*rac*-**1c**, 20 mM) and cis-1,2-cyclohexanedimethanol (**2b**, 10

mM) also in a 30 mL reaction vessel (1 mL total reaction volume) under the same reaction conditions used in Figure 5. The ketone decreased to 6.3 mM and 4.3 mM in 24 and 48 h, respectively. The diol concentration was 1.9 mM after 24 h and no diol was detected in 48 h. Overall, 17.5 mM normal lactone (**3b**) and 7.5 mM abnormal lactone (**3c**) (2.3:1, N:ABN) was achieved after 48 h. The *ee* of the normal lactone (**3b**) decreased from 61% (3a*S*,7a*S*) (24 h) to 39% (3a*S*,7a*S*) (48 h). The abnormal lactone (**3c**) was achieved with an *ee* of >99% (3a*R*,7a*S*), independent of the reaction time. The analytical lactone yield of the reaction was 86% after 48 h. The production of abnormal and normal lactones may rise a question regarding downstream processing, which has to be taken into account for technical scale applications. However, the above shown synthesis of chiral lactones was a proof-of-concept, which necessitates the formation of one lactone product in the cascade for a straightforward product work-up.

In summary, we have demonstrated NADH-dependent redox-neutral convergent cascades for lactonizations with type II flavin-containing monooxygenase from *Rhodococcus jostii* RHA1 (FMO-E) and the well studied HLADH. Compared to the previously developed convergent cascade with $CHMO_{Acineto}$ and TeSADH (ADH from *Thermoanaerobacter ethanolicus*)[3], we observed higher lactone yields (up to 90%). The main motivation of using NADH instead of NADPH in the redox-neutral cascade has been successfully demonstrated in two model systems. In the here presented convergent cascade, HLADH-catalyzed the oxidation of 1,4-butanediol promoting the conversion of cyclobutanone to *γ*-butyrolactone by the regeneration of NADH and also converging to the same product. Fused cyclobutanones and corresponding diols could also be applied in this system to synthesize normal and abnormal lactone products.

Our future work will focus on more controlled reaction conditions with respect to the molecular oxygen amount for higher product yields. The substrate scope of FMO-E is currently limited to cyclobutanones and the enzyme is quite unstable (thermally and under long-term operations). Hence, future studies focussing on solving its crystal structure, expanding the substrate scope of FMO-E, and improving its stability through protein engineering and immobilization are being carried out.

Experimental Section

All the chemicals were purchased from Sigma-Aldrich or Carl Roth and used as received except that bicyclo[4.2.0]octan-7-one and octahydrobenzofuran-2-one were synthesized as described in the Supporting Information. FMO-E was purified with Strep-tag® system (Figure S1, Table S1) while HLADH was purified with Ni-NTA purification system (Figure S2, Table S2). Protein concentration was determined by BCA protein quantification kit (PierceTM) from Thermo Scientific. All the enzymatic reactions were analyzed using gas chromatography (Table S4, Figure S12–S15). All the reactions were performed with purified enzymes. The details on the cultivation of the cells, overexpression, activity assays and analytics were given in the Supporting Information.

Reaction cascades (Figure 3 and Figure 4) involving FMO-E and HLADH were run in a total reaction volume of 1.0 mL in 30 mL glass vial consists of 20/100 mM ketone substrates, 10/50 mM diol substrates, 1.0 mM NAD^+, 1.0/2.0 U of FMO-E, 1.0/2.0 U of HLADH, 100 mM Tris-HCl (pH 8.0). The reactions were performed at 20°C with 180 rpm shaking speed. Samples (25 µL) were taken at the indicated time intervals through extraction (250 µL EtOAc with 2 mM methyl benzoate as the internal standard). After centrifuging (13,000 rpm; 1 min) and separating the two phases, the EtOAc layer was dried with $MgSO_4$, and then transferred to GC vials and analyzed by GC.

Reaction cascade (Figure 5) involving FMO-E and HLADH were run in a total reaction volume of 1.0 mL in 1.5 mL glass vial consists of 20 mM ketone substrate, 10 mM diol substrate, 1.0 mM NAD^+, 1.0 U of FMO-E, 1.0 U of HLADH, 100 mM Tris-HCl (pH 8.0). The reaction was performed at 20°C with 900 rpm shaking speed and samples were handled as described above.

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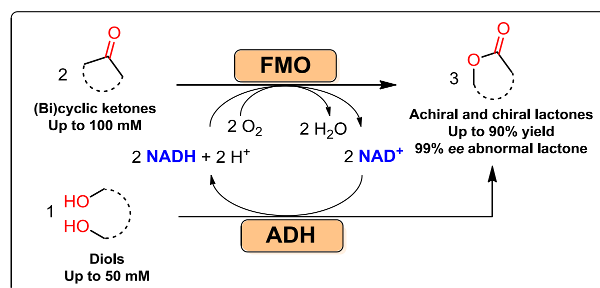
UPDATE

Nicotinamide Adenine Dinucleotide-Dependent Redox-Neutral Convergent Cascade for Lactonizations with Type II Flavin-Containing Monooxygenase

A recently discovered type II flavin-containing monooxygenase (FMO) and an alcohol dehydrogenase (ADH) were coupled for the synthesis of lactones (achiral and chiral) in a convergent cascade fashion using nicotinamide adenine dinucleotide (NADH) as cofactor. Two molar equivalents of ketones and one molar equivalent of corresponding diols were catalyzed by FMO and ADH respectively to the same lactone or normal and abnormal lactones, whereby the NADH regeneration could be achieved by the oxidation of ketone and diol. The cascade reaction is atom efficient and self-sufficient system with respect to the nicotinamide cofactor. The monooxygenase that accepts NADH presents high potential for Baeyer-Villiger oxidations.



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