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Enhanced Ene-Reductase Activity through Alteration of Artificial Nicotinamide Cofactor Substituents

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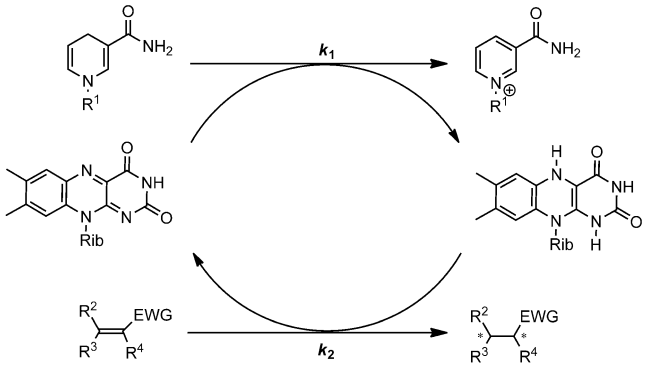
The reduction of activated C=C double bonds is an important reaction in synthetic chemistry owing to the potential formation of up to two new stereogenic centers. Artificial nicotinamide cofactors were recently presented as alternative suppliers of hydride equivalents needed for alkene reduction. To study the effect of cofactors on the reduction of activated alkenes, a set of *N*-substituted synthetic nicotinamide cofactors with differing oxidation potentials were synthesized and their electrochemical and kinetic behavior was studied. The effects of the synthetic cofactors on enzyme activity of four ene reductases are outlined in this study, where the cofactor mimic with an *N*-substituted 4-hydroxy-phenyl residue led to a sixfold higher v_{max} relative to the natural cofactor NADH.

Introduction

Ene reductases are interesting alternatives for organic chemists to chemical methods for the enantioselective reduction of activated triple or double bonds.[1,2] Ene reductases have proved to be excellent catalysts in terms of selectivity,[3] stability,[4] and tolerance towards enzyme engineering.[5] They are able to stereoselectively reduce double bonds in conjugation with aldehydes,[6] ketones,[7] nitroalkenes,[8] maleimids,[9] dicarboxylic acids,[10] esters,[11] and nitriles.[12] Ene reductases rely on a two-step ping-pong-bi-bi mechanism (Scheme 1). The flavin mononucleotide (FMN)-containing active site first binds the nicotinamide cofactor NAD(P)H, which reduces the prosthetic flavin. In a second step, the substrate binds to the active site and a hydride is transferred from the flavin to the β-carbon of the double bond in a Michael-type addition, leading to *ee* values of >99.5%.[13] The modification of the necessary nicotinamide cofactor reaches back to the 1930s. Karrer and co-workers identified the nicotinamide moiety of the NADPH as the active, hydride transferring part of the molecule for ene-reductase-catalyzed reactions.[14] This result implies that the adenine dinucleotide residue is mostly utilized for the recognition and positioning of the cofactor.[15] Previous work described several ene reductases to be promiscuous with regards to their specificity towards NADH and NADPH.[4,14,16–19] Owing to this cofactor promiscuity, different architectures of the hydride donor are feasible alternatives[20,21] as well as employing H_2O with a TiO_2-based photocatalyst as a hydride donor,[22] and the utilization of an electrode system with methyl viologen as a mediator.[23] The alteration of the nicotinamide function has, moreover, been studied with ene reductases. Recent work revealed two predominant positions for modification of the nicotinamide ring.[24] First, the amide function was replaced by other electron-withdrawing groups such as cyanide and carboxyl. Second, the adenine dinucleotide moiety was replaced by various other aliphatic and benzylic groups. With these simplified cofactors, conversions using natural NADH could be matched. In contrast to flavin-containing enzymes, the direct hydride transfer from the cofactor to the substrate with alcohol dehydrogenases was not feasible when cofactor mimics were applied. This enabled the bio-selection in crude cell lysate, where competing reduction reactions of alcohol dehydrogenase and ene reductase take place.[24] As yet, there has not been an example of a significantly enhanced enzyme activity with altered cofactors. By means of kinetic investigations using the ene re-



Scheme 1. Reaction scheme of a flavin-mediated reduction of an activated C=C double bond. The first step is limited by the kinetic constant k_1 and leads to a reduced flavin. The second step is the reduction of substrate with the kinetic constant k_2. If $k_1 < k_2$, then the flavin reduction is the rate limiting step.

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ductase, old yellow enzyme 1 (OYE1), the flavin-reducing step (k_1) was identified to be slower than the substrate reduction (k_2).[25] Therefore, a faster flavin reduction should lead to an increased overall reaction rate (Scheme 1). We reasoned that by varying the electrochemical potential of the utilized cofactor, an improved k_1 might be observed. Aromatic residues attached to the endogenous nitrogen atom could function as electron-donating residues and alter the electrochemical properties. In the present paper, we discuss the effect of cofactor potentials by substituting the adenine dinucleotide with different aromatic residues and the consequent influence on activity by using four ene reductases. The electrochemical properties of these cofactors were measured by cyclic voltammetry. Kinetic experiments revealed a sixfold increase in enzyme activity.

Results and Discussion

Cofactor design

The so-far investigated cofactors have in common that only sp^3-hybridized carbon atoms have been attached to the heterocyclic amine. The direct coupling of an aromatic system carrying various substituents to the nicotinamide ring offers many possibilities to alter the steric and electrochemical behavior of the cofactor. We synthesized the cofactors **2** and **4** by using Zincke's salt (3-carbamoyl-1-(2,4-dinitrophenyl)pyridine-1-ium-chloride) and named them similarly to the already described 1-benzyl-1,4-dihydro-nicotinamide (BNAH). Thus, PNAH relates to 1-phenyl-1,4-dihydro-nicotinamide and HPNAH relates to 1-(4-hydroxyphenyl)-1,4-dihydro-nicotinamide (Figure 1). The already described BNAH was synthesized as published previously and used for comparison.[24,26–28]

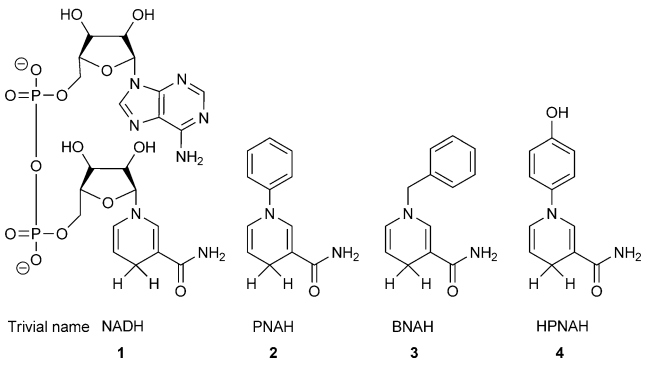


Figure 1. The set of cofactors used in this work. The trivial names are acronyms: NADH stands for nicotinamide adenine dinucleotide, PNAH for 1-phenyl-1,4-dihydro-nicotinamide, BNAH for 1-benzyl-1,4-dihydro-nicotinamide, and HPNAH for 1-(4-hydroxyphenyl)-1,4-dihydro-nicotinamide.

Cyclic voltammetry

The synthesized cofactors were studied by cyclic voltammetry to determine their electrochemical potentials and compare them to NADH. All measured compounds exhibited an irreversible oxidation peak at different potentials (Figure 2). As expected from the Hammett constants[29,30] for the inductive and mesomeric substituent effects, PNAH behaved similarly to NADH. HPNAH—which carries an additional *para*-hydroxy group—showed the lowest oxidation potential. A lower oxidation potential could indicate a higher ability for hydride donation.[31] BNAH, possessing only an inducing effect from the methylene group, revealed a higher oxidation potential than HPNAH and a lower one than NADH. After the first oxidation, a new process around −1 V was observed, which was assigned to a decomposition product as it was not observed when starting the measurements with the reduction step. PNAH and HPNAH showed a difference of 0.2 V between their oxidation potentials, which was highly suitable for studying the influence of the electrochemical potential of the cofactor. The difference between our values and those in the literature is due to an altered experimental setup.[32–35]

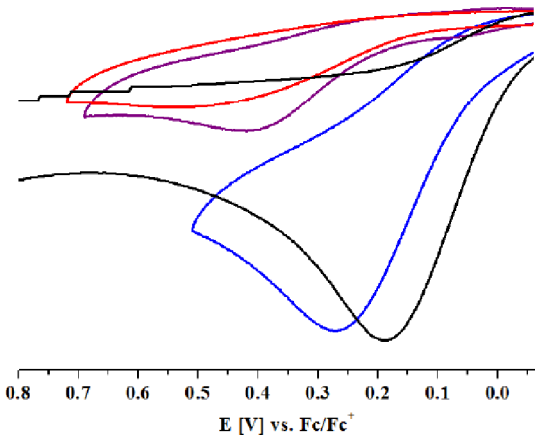
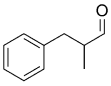
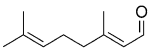
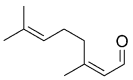


Figure 2. Overlapped cyclic voltammograms of NADH and the synthetic analogs BNAH, PNAH, and HPNAH. Measured in 0.1 M Bu_4NClO_4 solutions in DMSO with a glassy carbon working electrode, Pt counter electrode, and Ag/AgCl reference. The ferrocene/ferrocenium (Fc/Fc^+) couple served as an internal reference. Red refers to NADH, blue refers to BNAH, purple refers to PNAH, and black refers to HPNAH.

Enzyme screening

As the altered electronic properties of the cofactor derivatives BNAH, PNAH, and HPNAH could influence their interactions with the prosthetic flavin group of ene reductases, we tested four different enzymes. The employed reductases were the morphinone reductase from *Pseudomonas putida* M10 (MR),[36] the NAD(P)H-dependent 2-cyclohexen-1-one reductase from *Zymomonas mobilis* (NCR),[37] and the two old yellow enzymes OYE1 and OYE3 from *Saccharomyces pastorianus*.[38] These four proteins were screened with the four cofactors **1**–**4** and the substrates 2-methyl-2-pentenal (**5**), 2-methyl-cinnamaldehyde (**6**), and the isomers (*E*)-citral (**7**) and (*Z*)-citral (**8**). OYE1 and OYE3 both accepted the synthetic cofactors and the best conversions were achieved with BNAH (Tables S3 and S4 in the Supporting Information). OYE1 and OYE3 were employed in a threefold excess relative to MR and NCR. The conversions of OYE1 and OYE3 did not match the product formation of NCR. This proves the literature-known excellent activity of NCR.[39] MR displayed a preference for NADH for all substrates (Table S2 in the Supporting Information). NCR turned out to be

Table 1. Conversions [%] with different combinations of substrates and cofactors catalyzed by NCR.[a]

Substrates		NADH	PNAH	BNAH	HPNAH
	**5**	68.6 ± 1.2	53.7 ± 5.0	54.2 ± 2.6	82.0 ± 3.5
	**6**	68.7 ± 1.1	65.2 ± 4.8	66.1 ± 2.7	95.6 ± 3.1
	**7**	33.0 ± 0.5	74.0 ± 1.0	68.0 ± 1.6	91.6 ± 6.9
	**8**	46.8 ± 9.5	62.3 ± 7.8	51.3 ± 6.3	88.9 ± 9.3

[a] Reaction conditions: the substrate stock solution (10 μL, 100 mM in DMSO, final concentration = 2 mM) and cofactor stock solution (10 μL, 125 mM in DMSO, final concentration = 2.5 mM) were added to the enzyme solution (480 μL, 50 μg mL^{-1}, citrate buffer at pH 7.5, 12 mM). The mixture was stirred for 20 min at 30 °C.

the most active enzyme in the tested set, converting all substrates > 33% with the four cofactors. The best cofactor was HPNAH for all substrates, with excellent conversions of > 82% after 20 min (Table 1). Previous work on NCR with the natural cofactor NADH already showed a preference for the smaller substrates **5** and **6** (conversions > 68%) and lower conversions (< 47%) for the citral isomers **7** and **8**.[39] Interestingly, the synthetic cofactors did not display significantly lower activity with any of the substrates.

Kinetic experiments

Because of the significant difference in the oxidation potential of NADH and HPNAH in combination with the distinguished reactivities with NCR and (*Z*)-citral **8**, we performed additional kinetic studies. As the reaction proceeds through a ping-pong-bi-bi mechanism, the cofactor as well as (*Z*)-citral **8** had to be considered as substrates. Therefore, one component—either the cofactor or the substrate—was varied in the range 0.1–10 mM while the other was kept constant at 1 mM. As HPNAH does not exhibit a distinguishable wavelength in either its reduced or oxidized form, stopped-flow monitoring was not performed.

The samples were hence extracted after 5, 10, 15, 20, and 30 min, respectively. The amount of product was thereafter determined by GC-FID. The advantage of this approach in comparison to spectrometric determination of cofactor depletion is the facile reaction setup. As NADH can be oxidized by oxygen, the spectrometric setup requires a protecting gas. This is not necessary in our approach owing to the direct detection of the formed product rather than the indirect measurement of the absorption decrease of the cofactor. The kinetic data revealed that the reduction is dependent on the concentration of each varied component (Figure 3). The variation of NADH concentration showed an expected converging increase of turnover frequency at higher amounts. HPNAH acted substantially different to NADH and showed inhibition at concentrations larger than 0.4 mM. Owing to its low solubility in water, the HPNAH concentration was only varied between 0.1 and 5 mM. Impressively, a threefold increase in v_{max} was measured for HPNAH compared with that for NADH (Table 2). The reduction using a constant concentration of NADH suffers a strong inhibition by (*Z*)-citral **8**. The Michaelis constant, K_m, and the inhibition constant, K_i, for NADH were calculated to be 0.061 mM and 0.499 mM, respectively. The variation of NADH showed the expected behavior of an increased reaction rate at higher concentrations although the enzyme showed a low affinity towards NADH (K_m = 3.802 mM). With the utilization of HPNAH, the situation is inverted. The variation of the HPNAH concentration showed cofactor inhibition with a K_i value of 1.888 mM whereas a high (*Z*)-citral **8** concentration marginally inhibited the reaction rate with a K_i value of 14.897 mM. Additionally, the v_{max} value of NADH in both systems is much lower than v_{max} with HPNAH. These results indicate some guidelines for an applied reaction setup. The NCR–NADH system demands high cofactor concentrations as well as low (*Z*)-citral **8** concentrations. Both characteristics are unfavorable with respect to the needs of an efficient synthesis. As cofactors are usually combined with a recycling system, the apparent cofactor concentration is typically

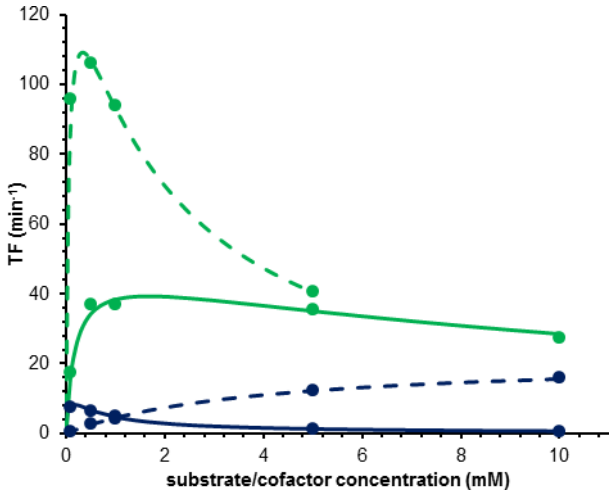


Figure 3. Turnover frequency (TF) per minute with varied substrate and cofactor concentrations. The blue curves refer to the systems comprising NADH and the green lines refer to the systems comprising HPNAH. Solid lines represent (*Z*)-citral **8** variation and the dashed lines represent the cofactor variation

Table 2. Kinetic parameters of the reduction reaction of (*Z*)-citral **8** catalyzed by the enzyme NCR.

	NADH		HPNAH	
	NADH const.[a]	*Z*-citral **8** const.[b]	HPNAH const.[a]	*Z*-citral **8** const.[b]
K_m [mM][c]	0.067	3.802	0.187	0.059
v_{max} [μmol min^{-1}][d]	0.305	0.454	1.012	3.124
K_i [mM][e]	0.499	–	14.897	1.888

[a] Reaction conditions: the substrate stock solution (5 μL, 40 mM in DMSO, final concentration = 1 mM) and cofactor stock solution (10 μL, in DMSO, final concentrations = 0.1/0.5/1/5/10 mM) were added to the enzyme solution (380 μL, 2 μg mL^{-1}, citrate buffer at pH 7.5, 12 mM). The mixtures were stirred for 5/10/15/20/30 min at 30 °C. [b] Reaction conditions: the cofactor stock solution (5 μL, 40 mM in DMSO, final concentration = 1 mM) and substrate stock solution (10 μL, in DMSO, final concentrations = 0.1/0.5/1/5/10 mM) were added to the enzyme solution (380 μL, 2 μg mL^{-1}, citrate buffer at pH 7.5, 12 mM). The mixtures were stirred for 5/10/15/20/30 min at 30 °C. [c] K_m = the Michaelis constant. [d] v_{max} = maximal velocity. [e] K_i = substrate inhibition constant.

low, which would result in low activity of the enzyme. In the NCR–HPNAH system, however, high substrate concentrations (10 mM) did not lead to a significant inhibition effect and hence allow increased substrate titres. The tolerance of high (*Z*)-citral **8** concentrations in addition to the low K_m of HPNAH renders the system significantly more efficient than the natural one.

An explanation for the observed increase in reaction speed could be the ability of HPNAH to reduce the flavin faster than NADH as a result of its geometry and potential. Massey et al. identified a 20 times slower flavin reduction in OYE1 than the subsequent reduction of cyclohexanone, which they used as the substrate in their study.[25] In our system, both molecules—the cofactor and the substrate—use the same active site and are competing for binding. The combination of low steric hindrance and a low oxidation potential seems to allow HPNAH to perform an accelerated reductive half reaction (k_1).

Conclusions

The variation of the nicotinamide substituents led to a significantly altered behavior. The screening of the four enzymes NCR, MR, OYE1, and OYE3 revealed the highly active cofactor-enzyme pair NCR–HPNAH. Cyclic voltammetric measurements confirmed the lowered oxidation potential of HPNAH, representing a higher ability for hydride donation. This provides a possible explanation for the increased activity. The performed kinetic experiments further show that the NCR–HPNAH system is beneficial for the synthesis of the product citronellal. The ease of preparation and the far-reaching effects of the aromatic substituted nicotinamide cofactors provide an addition to the biocatalytic toolbox. This can be perceived as a chemical approach to synthetic biology. We term this chemical strategy "ChemBricks" in analogy to BioBricks. The use of chemical modification for biological challenges is still a niche area. This niche has high potential to be applied to cofactors and co-substrates such as flavins, hemes, thiamines, α-ketoglutarates, and pyrroloquinoline quinones (PQQ).

Experimental Section

Chemicals and analytics

Solvents and buffer components were obtained from Sigma–Aldrich (Schnelldorf, Germany). Non-commercially available chemicals such as the synthetic cofactors were synthesized as described below. Analytics were carried out on a Shimadzu GC-2010-system (Kyōto, Japan) with a flame ionization detector (FID) or a mass spectrometer (MS). Helium (MS) and hydrogen (FID) were used as carrier gases with a flow of 30 cm s^{-1} and an injection temperature of 250 °C. The injection volume was 1 μL with a split of 1:5. Quantification was achieved by a calibration curve and the internal standard 1-octanol.

Cofactor synthesis

BNAH (**3**) was synthesized as previously described.[24]
^{1}H NMR (250 MHz, CDCl3): δ = 3.18 (s, 2H), 4.31 (d, 2H), 4.75 (m, 2H), 5.33 (s, 2H), 5.75 (d, 1H), 7.32 ppm (m, 5H).
The syntheses of PNAH (**2**) and HPNAH (**4**) were carried out in three steps. The first step was the preparation of Zincke's salt (3-carbamoyl-1-(2,4-dinitrophenyl)pyridiniumchloride). Therefore, nicotinamide (7.32 g, 59.9 mmol) and 2,4-dinitrochlorobenzene (13.35 g, 65.91 mmol) were stirred for 30 min at 100 °C without a solvent. After cooling to room temperature, the resulting orange glassy solid was dissolved in methanol (80 mL). Then, methyl *tert*-butyl ether (MTBE) (50 mL) was added, the mixture was shaken, and the upper layer was discarded. This step was repeated twice. The precipitated solid was filtered, dissolved in methanol (20 mL), and ethyl acetate (100 mL) was added. The solvent was discarded and the residual solid was dried under vacuum and stored under nitrogen. ^{1}H NMR (250 MHz, D_2O): δ = 8.27 (d, 1H), 8.49 (t, 1H), 8.96 (d, 1H), 9.34 (m, 3H), 9.69 ppm (s, 1H).
The second and the third steps were carried out subsequently without intermediate purification. 3-Carbamoyl-1-(2,4-dinitro-phenyl)pyridiniumchloride (1 g, 3.08 mmol) was dissolved in methanol (150 mL). Then, anilin (PNAH) or 4-amino-phenol (HPNAH; 3.08 mmol) were added. The obtained deep-red solution was heated at 50 °C until it turned yellow. Then, the solvent was evaporated and the solid was dissolved in H_2O. The obtained solution was extracted three times with MTBE (50 mL). Then, $NaHCO_3$ (0.5 g) was added and a nitrogen atmosphere was applied. Over the course of 1 h, $Na_2S_2O_4$ (2.3 g, 10.7 mmol) was added in portions. In the case of HPNAH, the aqueous layer was laminated with MTBE to ensure continuous product extraction. After the complete addition of $Na_2S_2O_4$, the solution was extracted three times with MTBE (50 mL). The solvent was evaporated and the obtained solid was stored under nitrogen.
PNAH: ^{1}H NMR (500 MHz, DMSO): δ = 3.21 (d, 2H), 4.97 (m, 1H), 5.52 (s, 2H), 6.31 (m, 1H), 7.10 (m, 3H), 7.35 (m, 2H), 7.54 ppm (t, 1H).
HPNAH: ^{1}H NMR (500 MHz, DMSO): δ = 3.01 (d, 2H), 4.83 (m, 1H), 6.28 (m, 1H), 6.85 (m, 5H), 7.14 (m, 2H), 9.35 ppm (s, 2H).

Cyclic voltammetry

Cyclic voltammetry was carried out in 0.1 M Bu_4NClO_4 solutions by using a three-electrode configuration (glassy carbon working electrode, Pt counter electrode, Ag/AgCl reference) and a PAR 273 potentiostat and function generator. Argon was used as the inert gas und DMSO was used as the solvent. The ferrocene/ferrocenium (Fc/Fc^+) couple served as the internal reference.

The exemplary oxidation peaks of the cofactors PNAH, NADH, BNAH, and HPNAH are shown in Figure 2.

Enzyme preparation

For the protein expression, *E. coli* BL21(DE3) was transformed with the corresponding vectors (NCR, MR, OYE1, and OYE3). Then, a single colony was transferred to 5 mL of an overnight culture. Terrific broth (TB) medium (400 mL) was inoculated with overnight culture (4 mL) and incubated until an OD_{600} of 0.6 was reached. Then, isopropylthiogalactopyranoside (IPTG, 0.1 mM) was added for induction. Cells were harvested after incubation overnight at 30 °C and 180 rpm and resuspended in 50 mM potassium phosphate buffer at pH 7.5. Cells were disrupted by sonication on ice (4×1 min, 1 min intervals). The cell debris was removed by centrifugation (37 000 g, 45 min, 4 °C) and the supernatants were recovered. Purification was carried out with 1 mL His GraviTrap TALON columns and 150 mM imidazole solution as eluent. One dialysis against tris(hydroxymethyl)aminomethane (TRIS) buffer (50 mM, pH 7.5) for 1 h and overnight at 4 °C ensured the absence of imidazole in the purified enzyme solutions.

Substrate screening

A stock solution of the substrate (10 μL, 100 mM in DMSO, end concentration = 2 mM) and cofactor stock solution (10 μL, 125 mM in DMSO, end concentration = 2.5 mM) were added to the enzyme solution (480 μL, NCR/MR: 50 μg mL^{-1} and OYE1/OYE3: 150 μg mL^{-1} in citrate buffer at pH 7.5, 12 mM). The mixture was stirred for 20 min at 30 °C und 180 rpm. Then, the samples were extracted with MTBE (500 μL) and analyzed by GC-FID.

Kinetic experiments

Cofactor variation: A (*Z*)-citral stock solution (10 μL, 40 mM in DMSO, end concentration = 1 mM) and cofactor stock solution (10 μL, in DMSO, end concentration = 0.1/0.5/1/5/10 mM) were added to the enzyme solution (380 μL, NCR: 2 μg mL^{-1} in citrate buffer at pH 7.5, 12 mM). The mixtures were stirred for 5/10/15/20/30 min at 30 °C and 180 rpm. Afterwards, the samples were extracted with MTBE (400 μL) and analyzed by GC-FID.

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Keywords: asymmetric reduction · biocatalysis · cofactors · ene reductase · enzyme kinetic

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