Mimicking Nature: Synthetic Nicotinamide Cofactors for C=C Bioreduction Using Enoate Reductases

Caroline E. Paul,[§,†] Serena Gargiulo,[§,‡] Diederik J. Opperman,[‖] Iván Lavandera,[†] Vicente Gotor-Fernández,[†] Vicente Gotor,[†] Andreas Taglieber,[ǀ] Isabel W. C. E. Arends[‡] and Frank Hollmann*[,‡]

Department of Biotechnology, Delft University of Technology, Julianalaan 136, 2628BL Delft, The Netherlands, Department of Biotechnology, University of the Free State, Bloemfontein 9300, South Africa, Department of Organic and Inorganic Chemistry, University of Oviedo, Calle Julián Clavería 8, 33006 Oviedo, Spain, and Firmenich SA, Corporate R&D, Biotechnology&Bioengineering, Route des Jeunes 1, CH-1211 Geneva 8, Switzerland

f.hollmann@tudelft.nl

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ABSTRACT
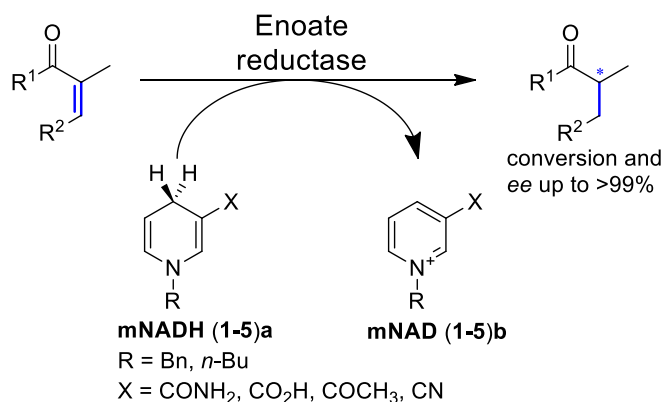
A series of synthetic nicotinamide cofactors were synthesized to replace natural nicotinamide cofactors and promote enoate reductase (ER)-catalyzed reactions without compromising activity or stereoselectivity of the bioreduction process. Conversions and enantioselectivities of up to >99% were obtained for C=C bioreductions and the process was successfully upscaled. Furthermore, high chemoselectivity was observed when employing these nicotinamide cofactor mimics (mNADs) with crude extracts in ER-catalyzed reactions.

The asymmetric reduction of conjugated C=C double bonds using enoate reductases (ERs, EC 1.3.1.31) is receiving great interest in preparative organic chemistry.[1]

The rapidly expanding scope of ER-catalyzed

[§] Both authors contributed equally.
[†] University of Oviedo.
[‡] Delft University of Technology.
[‖] University of the Free State.
[ǀ] Firmenich SA.

(1) (a) *Enzyme Catalysis in Organic Synthesis*, (Eds.: Drauz, K.; Gröger, H.; May, O.), 3rd ed., Wiley-VCH, Weinheim, **2012**; (b) Hollmann, F.; Arends, I. W. C. E.; Holtmann, D.; *Green Chem.* **2011**, *13*, 2285-2313; (c) Faber, K. *Biotransformations in Organic Chemistry*, 6th ed., Springer, Berlin, **2011**; (d) Toogood, H. S.; Gardiner, J. M.; Scrutton, N. S. *ChemCatChem* **2010**, *2*, 892-914; (e) Stürmer, R.; Hauer, B.; Hall, M.; Faber, K. *Curr. Opin. Chem. Biol.* **2007**, *11*, 203-213.
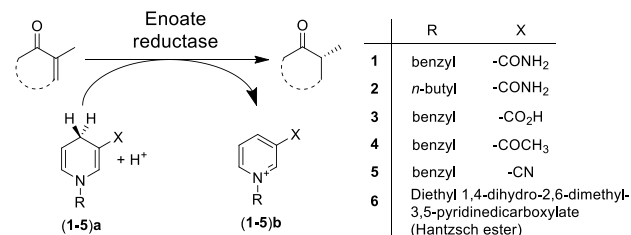
stereospecific reductions makes this method a viable alternative to transition metal-catalyzed reductions. New ERs are constantly added to the toolbox from natural sources[1d,2] and obtained *via* protein engineering.[3] These enzymes are *en route* to becoming truly practical catalysts, although one remaining challenge involves their dependency on reduced nicotinamide cofactors [NAD(P)H] providing the reducing equivalents needed for the alkene bioreduction. NAD(P)H is rather expensive,[4] forbidding its stoichiometric use at high scale. In principle, this can be overcome by using an (enzymatic) cofactor regeneration system.[5]

Another challenge resulting from the NAD(P)H-dependency of ERs arises with conjugated aldehydes and ketones as starting materials. Here, frequently unsatisfactory chemoselectivity is observed unless highly purified, alcohol dehydrogenase (ADH)-free, enzyme preparations are used. The reason thereof lies in the overlap of the substrate scope for both enzyme classes. As a result, both substrates and products of the ER-catalyzed transformation can also be converted by 'contaminating' ADHs leading to complex product mixtures, impairing the overall chemoselectivity of the reactions.[1b,6]

Substitution of NAD(P)H as a reducing agent by other reductants appears to be straightforward with ERs and may be the method of choice to circumvent the above-mentioned challenges. Indeed, some promising approaches for NAD(P)H-independent regeneration have been reported recently.[5a,7]

We became interested in synthetic, functional mimics of the natural nicotinamide cofactors (mNAD, Scheme 1) as stoichiometric reductants to promote ER-catalyzed reduction reactions.

Scheme 1. Asymmetric bioreduction of conjugated C=C double bonds using synthetic nicotinamide mimics (**1-5**)**a** and **6**

	R	X
1	benzyl	$-CONH_2$
2	*n*-butyl	$-CONH_2$
3	benzyl	$-CO_2H$
4	benzyl	$-COCH_3$
5	benzyl	$-CN$
6	Diethyl 1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate (Hantzsch ester)	

These mNADs are simple and cheap to synthesize, starting from commercially available pyridine derivatives (**1,3-5**)**c**; thus, the nitrogen was alkylated with benzyl or *n*-butyl bromide under reflux to obtain the bromide salts (**1-5**)**b** in high yields (81-92%), and the pyridinium ring was reduced into the corresponding dihydropyridine (**1-5**)**a** in moderate to high yields (35-81%) with sodium dithionite and sodium bicarbonate (Scheme 2).

Scheme 2. Straightforward two-step synthesis of the reduced nicotinamide mimics mNADs (**1-5**)**a**
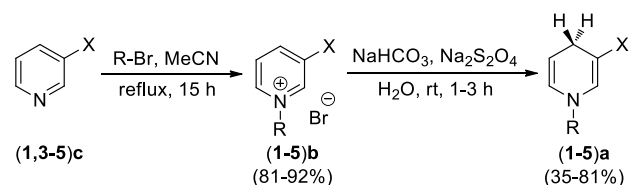
Synthetic mNADs have received considerable attention as cost-efficient alternatives to the natural NAD(P)H cofactors.[8] Unfortunately, the catalytic efficiencies of the wild-type alcohol dehydrogenases with mNADs generally fall back by orders of magnitude below their activity with the natural cofactors.[9] In that respect, ERs represent an exception as they exhibit significant 'cofactor promiscuity'.[7a-c] In a first set of experiments, we

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(9) This adds up in a biochemical sense as high specificity of enzymes for either phosphorylated or non-phosphorylated nicotinamide cofactors is essential for controlling cellular metabolism.

evaluated the scope of enzymes accepting the mNAD **1a** as a replacement for NAD(P)H. As a model reaction, we chose the reduction of ketoisophorone (**7a**) to the corresponding levodione product (**7b**) to assess the conversion as well as the enantioselectivity of the ER-catalyzed reaction (Table 1). We were pleased to find that **1a** could replace the natural cofactors with a range of ERs without impairing the final yield or enantiospecificity of the reaction (entries 1-9). It is worth mentioning here that in the absence of either cofactor or enzyme no conversion was detectable within the time frame of the experiments.

Table 1. Performance of **1a** as a replacement for NADH or NADPH in the asymmetric bioreduction of ketoisophorone (**7a**) with different ERs[a]
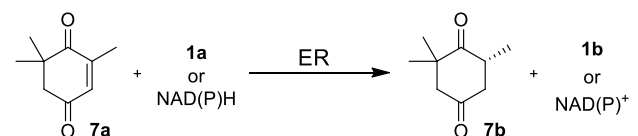
entry	ER[b]	cofactor	conversion (%)[c]	ee (%)[c]
1	YqjM	NADH	92	84 (R)
2		NADPH	90	87 (R)
3		**1a**	96	85 (R)
4	*Ts*ER	NADH	90	>95 (R)
5		NADPH	>99	>95 (R)
6		**1a**	>99	>95 (R)
7	*Rm*ER	NADH	76	95 (R)
8		NADPH	73	95 (R)
9		**1a**	72	96 (R)

[a] Conditions: [substrate]$_0$ = [cofactor]$_0$ = 10 mM, [ER] = 90-200 µg/mL, T = 30 °C, reaction time: 4 h; [b] YqjM: ER from *Bacillus subtilis*,[10] *Ts*ER: ER homologue from *Thermus scotoductus*,[11] *Rm*ER: ER from *Ralstonia metallidurans* CH34; [c] determined by GC analysis.

For further investigations the enoate reductase homologue from *Thermus scotoductus* (*Ts*ER) was used.[11] As shown in Table 2, a broad range of different enones (entries 1-8), enals (entries 9-12) and maleimides (entries 13-16) could be converted in excellent yield and enantiospecificity demonstrating the preparative broadness of the 'mimic-approach'. Currently, we are bringing the proposed bioreduction scheme to preparative scale. A first gram-scale reduction of *N*-phenyl-2-methyl maleimide (500 mM) gave excellent conversion and optical purities of the product (>99%) and acceptable isolated yields (>70%), showing the synthetic usefulness of this C=C reduction process to obtain enantioenriched compounds on a high scale.

In order to obtain a more detailed insight into the reduction performance, the mNADs (**1-5**)**a** and **6** were

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evaluated and compared to the natural cofactors (NADH and NADPH).

Table 2. Substrate scope of **1a**-driven chemoselective reductions of α,β-unsaturated carbonyl compounds (**7-14**)**a**[a]

entry	product	cofactor	conversion (%)[b]	ee (%)[b]
1	7b	NADH	>99	>95 (R)
2		**1a**	>99	>95 (R)
3	8b	NADH	93	>99 (S)
4		**1a**	82	>99 (S)
5	9b	NADH	>99	>99 (R)
6		**1a**	>99	>99 (R)
7	10b	NADH	>99	>99 (R)
8		**1a**	>99	>99 (R)
9	11b	NADH	>99	>99 (R)
10		**1a**	>99	>99 (R)
11	12b	NADH	21	n.d.[c]
12		**1a**	18	n.d.[c]
13	13b	NADH	>99	n.a.[d]
14		**1a**	>99	n.a.[d]
15	14b	NADH	>99	>99 (R)
16		**1a**	>99	>99 (R)

[a] Conditions: [substrate]$_0$ = [cofactor]$_0$ = 10 mM, [*Ts*ER] = 90 µg/mL, T = 30 °C, reaction time: 4 h; [b] Determined by GC analysis; [c] n.d. = not determined. [d] n.a. = not applicable.
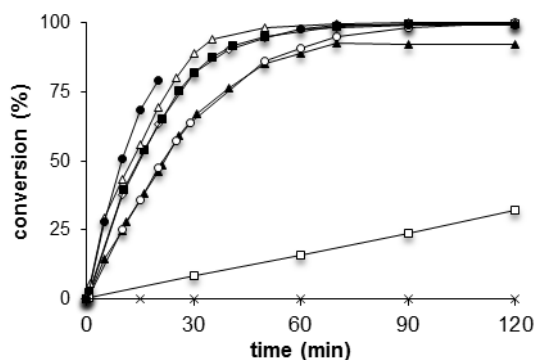

Figure 1. Comparison of enzymatic conversions for the reduction of ketoisophorone (**7a**) to (6*R*)-levodione (**7b**) catalyzed by *Ts*ER using different cofactors. Conditions: [ketoisophorone]$_0$ = [cofactor]$_0$ = 10 mM, [*Ts*ER] = 100 µg/mL = 2.2 µM in MOPS buffer (50 mM, pH 7 with 5 mM CaCl$_2$, containing 2% v/v MeOH in the case of (**1-5**)**a** and **6**). **1a**:◇, **2a**:●, **3a**:△, **4a**: ○, **5a**: □, **6**: X, NADH: ▲, NADPH: ■.

Figure 1 shows the time courses of these bioreduction reactions. With the exception of the nitrile analogue (**5a**)

and the Hantzsch ester (**6**), all synthetic nicotinamides exhibited equal or better activity with *Ts*ER. For example, initial rates obtained with **2a** were 1.5-fold higher than with NADPH and almost double than with NADH. It is worth mentioning that the enantioselectivity in all cases was exclusive. The observed high activity of mimics (**1-4**)**a**, even exceeding the activity of the natural cofactors, was somewhat unexpected, as with other enzyme classes investigated so far a significant decrease in activity had been observed. Molecular docking simulations of *Ts*ER with the mimics (Figure 2) confirmed the existence of productive binding modes for mimics **1a** to **5a**, leading to the reduced catalytically active $FMNH_2$ species.[12] Interestingly, for the Hantzsch ester (**6**) only unproductive binding was observed, supporting the experimental observations. Possibly, the increased activity with mimics can be explained by an increased FMN-reduction rate.
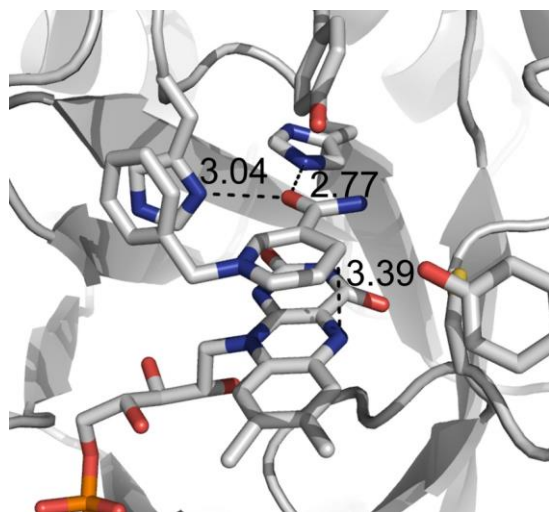

Figure 2. Exemplary result of docking **1a** into the active site of *Ts*ER.

Finally, we investigated the presumed chemoselectivity advantage of using NADH mimics over natural cofactors. For this, the product distribution in the reduction of citral (**15**) was compared using crude preparations of YqjM, recombinantly expressed in *Escherichia coli*. As shown in Table 3, using **1a** as a reductant, the desired C=C double bond reduction product (citronellal, **16**) was obtained as the sole product, while with NADH significant amounts of carbonyl reduction products (citronellol **18** and geraniol **17**) were formed.

Overall, we have demonstrated that cheap synthetic analogues of the natural nicotinamide cofactors (mNADs) represent a true alternative to the established regeneration systems to promote ER-catalyzed reduction reactions and apply them to preparative scale. Already under non-optimized conditions, equal or higher conversions have

been obtained compared to those with the natural cofactors, without altering the selectivity.

Table 3. Chemoselectivity of YqjM-crude extract-catalyzed reduction of citral (**7**) using NADH and **1a** as stoichiometric reductant
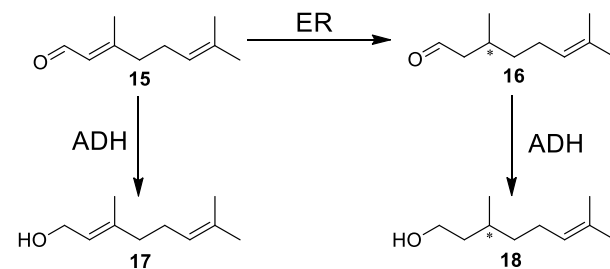
entry	cofactor	**16** (%)[a]	**17** (%)[a]	**18** (%)[a]	Selectivity (%)[b]
1	**NADH**	21	20	20	34
2	**1a**	18	<1	<1	>90

[a] Conversion determined by GC analysis. [b] C=C double bond reduction over C=O bond reduction.

Another very interesting opportunity with mNADs lies in the bioorthogonality[13] of these reduction schemes. The very poor activity of most enzyme classes tested so far with mNADs (particularly alcohol dehydrogenases and monooxygenases) enable the use of poorly purified (and hence cheap) ER preparations without impairing the selectivity of the desired reactions. Admittedly, issues such as the *in situ* regeneration of catalytic amounts of mNADs and/or recycling will have to be addressed in order to fully exploit their catalytic potential. Also, more mechanistic and kinetic studies as well as further modeling will be necessary to understand the catalytic mechanism. These studies are currently underway in our laboratories, eventually leading to a truly practical approach for *White Biotechnology*.

Supporting Information Available. Experimental procedures, full characterization and spectroscopic data for compounds (**1-5**)**a-b**, molecular docking and analytical methods. This material is available free of charge via the Internet at http://pubs.acs.org.

(12) Although at this stage we cannot entirely exclude a mechanism wherein FMN is reduced in solution and then binds to the apo-ER.

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