

# Efficient *in situ* Regeneration of NADH Mimics by an Artificial Metalloenzyme

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**ABSTRACT:** NADH mimics (mNADHs) have been shown to accelerate and orthogonally activate ene reductase-catalyzed reactions. However, existing regeneration methods of NAD(P)H fail for mNADHs. Catalysis with artificial metalloenzymes based on streptavidin (Sav) variants and a biotinylated iridium cofactor enable the mNADHs regeneration with formate. This regeneration can be coupled with ene reductase-catalyzed asymmetric reduction of  $\alpha,\beta$ -unsaturated compounds, due to the protective compartmentalization of the organometallic cofactor. With 10 mol% mNAD<sup>+</sup>, a preparative scale reaction (> 100 mg) gave full conversion with 98 % *e.e.*, where TTNs reached 2000 with respect to the Ir cofactor under ambient atmosphere in aqueous medium.

**KEYWORDS.** Artificial metalloenzyme, iridium, biocatalysis, renewable source, asymmetric catalysis

The increasing industrial use of biocatalytic processes can by-and-large be traced back to the high chemo-, regio-, and enantioselectivity of engineered enzymes.<sup>1-7</sup> These features enable low priced products thanks to high space time yields, reduced separation expenditures and waste formation. Oxidoreductases are firmly established as valuable tools for synthetic chemistry purposes. However, more than 400 enzymatic redox reactions require relatively costly pyridine nucleotide cofactors NAD(P)H as redox equivalents, typically in combination with an enzymatic recycling system.<sup>8-10</sup>

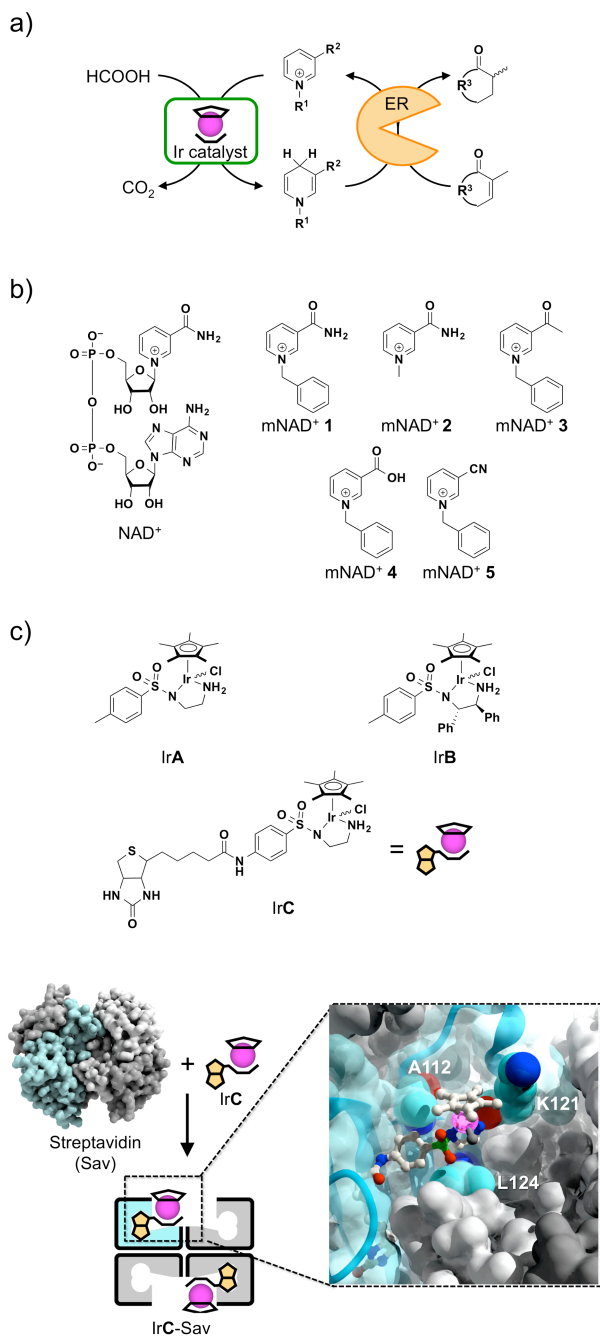
Various synthetic NAD(P)H mimics (mNADHs) have been synthesized for various purposes including: i) mechanistic probes,<sup>11-13</sup> ii) a decrease in the relatively expensive operational costs of NAD(P)H-dependent enzymes,<sup>14</sup> iii) the enhancement of the catalytic activity of selected NADH-dependent enzymes and iv) orthogonality against NADH as a limited number of enzymes accept mNAD(H) as cofactor (Scheme 1).<sup>14-20</sup> Simple mNADHs **1-5** (Scheme 1) might however suffer from various disadvantages, foremost the limited number of enzymes that accept them as redox equivalents. Further potential drawbacks include low solubility of the reduced form of the mimic and reduced chemical stability, if the mimics are electronically very different from natural NAD(P)H.

For *in situ* NAD(P)H regeneration, enzymes, such as alcohol dehydrogenases, glucose dehydrogenases and phosphite dehydrogenase, have been widely utilized.<sup>21,22</sup> However, these enzymatic methods cannot be applied to mNAD<sup>+</sup>s, as these do not accept mNAD<sup>+</sup> as a substrate (see iv) above). Recently an *in situ* catalytic reduction of mNAD<sup>+</sup> by a rhodium complex was reported, albeit at the cost of a low TTN.<sup>19</sup> To date, only few studies report the *in situ* regeneration of mNADHs which, has hampered their widespread use as redox mediators.<sup>14,16,23-27</sup> Regeneration challenges typically include: i) deactivation of the enzymatic partners and/or organometallic regeneration catalyst as well as ii) low productivity, selectivity and initial

activity.<sup>28-33</sup> The very limited success in mNADH regeneration severely hampers their widespread use and application.

In the past decade, artificial metalloenzymes (AMEs) have attracted attention as an alternative to both homogeneous catalysts and enzymes.<sup>34-43</sup> In this context we have shown that artificial transfer hydrogenases (ATHases) based on an abiotic biotinylated Ir-pianostool cofactor, IrC (Scheme 1c) and streptavidin variants, provide an attractive solution to the incompatibility of synthetic transition metal catalysts and enzymes.<sup>44-51</sup> Cascade reactions of ATHases with a range of different enzymes, namely a flavin dependent monooxygenase, heme dependent enzymes (catalase and peroxidase), a flavin dependent amine oxidase and amino acid oxidases were realized.<sup>52</sup> Since ATHases can reduce NAD(P)<sup>+</sup>,<sup>52,53</sup> we set out to explore the versatility of ATHases towards the regeneration of mNAD<sup>+</sup>s.

As an mNADH-accepting enzyme, the ene reductase (ER) of the Old Yellow Enzyme family from *Thermus scotoductus* (*TsOYE*) was selected.<sup>14,54</sup> Compared to the use of NAD(P)H, similar or even higher initial rates and conversions have been reported for reactions involving stoichiometric amounts of the mimic **1** in the presence of *TsOYE*.<sup>14,19</sup> ATHases were generated by employing a 1 : 2 ratio of the biotinylated cofactor and biotin binding sites.<sup>55</sup> As a stoichiometric reducing agent, sodium formate was selected (Scheme 1a). To identify an efficient regeneration system for the reduction of *N*-ethyl-2-methylmaleimide **6** (Scheme 2) with *TsOYE*, five different NAD<sup>+</sup> mimics **1-5** (Scheme 1b) were screened in combination with the biotinylated Ir-cofactor IrC and a range of Sav variants. Previous studies with the ATHase have revealed that mutations at three close lying positions (i.e. S112, K121 and L124) have a significant impact on the catalysis outcome (Scheme 1c).<sup>56</sup>

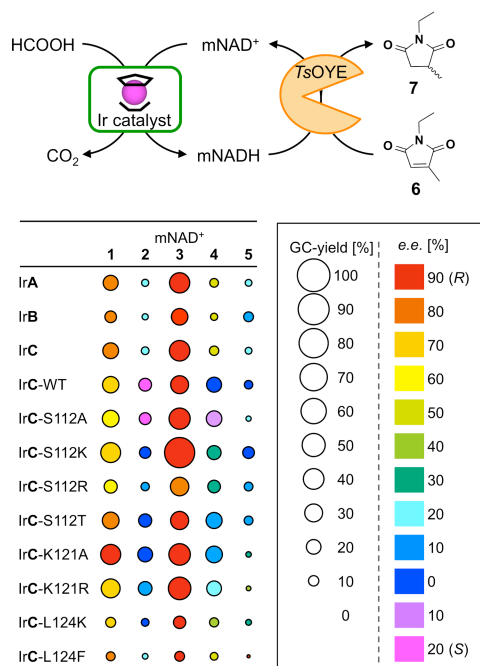


**Scheme 1.** (a) Catalytic reduction of Michael acceptors by ene reductase (ER) and concurrent catalytic cofactor regeneration by IrCp\* complexes. (b) Nicotinamide mimics 1-5 (mNAD<sup>+</sup>) tested as cofactors in this study. (c) IrCp\* complexes (IrA-C) and artificial transfer hydrogenase (IrC-Sav) tested for the reduction of mNAD<sup>+</sup> 1-5. Inset: close-up view of the structure of IrC-Sav S112A variant (PDB ID: 3PK2).

Although higher initial rates for mNAD<sup>+</sup> 1 reduction were observed under acidic conditions (Table S2), the pH of the reaction was set to 7.0. This value constitutes a trade-off between the pH-dependence of the ATHase activity and the mNADH stability (Figure S1). Furthermore, a neutral pH provides sufficient hydrolytic stability of the maleimide **6** used as

a model substrate.<sup>57,58</sup> No significant influence of formate on the activity of *TsOYE* was observed (Figure S2). For the screening, both the biotinylated Ir-cofactor IrC as well as non-biotinylated analogues IrA and IrB were included as catalysts (Scheme 1c).

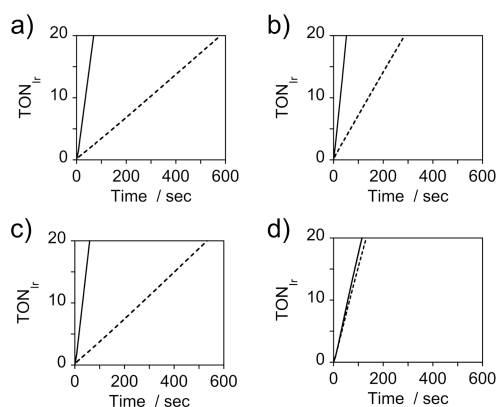
The fingerprint display summary (Scheme 2, Table S3) highlights the following features: a) mNADH **3** showed best performance in terms of enantioselectivity and GC-yield; b) four of the Sav variants with mNAD<sup>+</sup> **3** provided higher GC-yield and comparable activity to the protein-free cofactor IrC or its non-biotinylated analogues IrA and IrB; the best suited Sav variant is mutant S112K, providing over two-fold improvement compared to the protein-free Ir complexes; c) while it has been previously shown that stoichiometric amounts of mNADH **1** combined with *TsOYE* leads to the best GC-yield,<sup>14</sup> the mimic mNADH **3** proved most suitable (both in terms of *e.e.* and GC-yield) for the cascade including the Ir-based ATHase regeneration system (Figure S3)<sup>9,59,60</sup> and d) the nature of the mNADH plays a critical role in determining the *e.e.* of the reduction product **7**. We assume that the last point is due to the relative rates of reduction of mNAD<sup>+</sup> 1-5 vs. substrate **6** by the ATHase (vide infra).<sup>14</sup>



**Scheme 2.** Fingerprint display of the GC-yield and *e.e.* for *N*-ethyl-2-methylmaleimide **6** reduction by *TsOYE* / mNADHs 1-5 and IrCp\* / HCOOH based regeneration system. 10  $\mu$ M catalyst (IrCp\*), 0.5 mg/ml (3  $\mu$ M) *TsOYE*, 1 mM mNAD<sup>+</sup>, 10 mM *N*-ethyl-2-methylmaleimide **6**, and 2.0 M HCOONa in 0.6 M MOPS (pH 7.0); experiments were performed at 30 °C for 4 h on a 400  $\mu$ l scale. GC-yield and enantiomeric excess (*e.e.*) were determined by GC with benzyl alcohol as internal standard; GC-yields are based on product formation.

In order to evaluate the inactivation of the Ir-based regeneration catalyst in the presence of *TsOYE*, the initial rate of mNAD<sup>+</sup> **3** reduction was determined spectrophotometrically (Figure S4). The initial rate for the protein-free cofactor IrC is about 1.6 fold faster than for the best ATHase IrC-Sav S112K.

However, in the presence of the *TsOYE*, the relative activity is reversed: the protein-free cofactor IrC loses over 80% of its initial activity. In stark contrast, it maintains over 80% of its activity when incorporated into Sav S112K (Figure 1). The *TsOYE* maintains its activity in the presence of either IrC or IrC-Sav112K (Figure S5).



**Figure 1.** Initial rates of mNAD<sup>+</sup> **3** reduction catalyzed by (a) IrA, (b) IrB, (c) IrC, and (d) IrC-Sav S112K in the absence (solid line) and in the presence of *TsOYE* (dashed line) monitored by UV-vis absorption at 378 nm. 10 μM Ir-catalyst, 0.5 mg/ml (3 μM) *TsOYE*, 1 mM mNAD<sup>+</sup> **3**, and 2.0 M HCOONa in 0.6 M MOPS (pH 7.0); experiments were performed at 25 °C.

After identifying the combination of mNAD<sup>+</sup> **3**, IrC and Sav S112K as the most efficient artificial cofactor regeneration system, it was compared with the established regeneration method for reducing equivalents comprised of NAD(P)<sup>+</sup>, GDH and glucose (1.2 mg of GDH per ml). *TsOYE* mediated reduc-


tion coupled to the mNAD<sup>+</sup> **3**-ATHase system (10 μM, 1.2 mg of Sav per ml) gave higher GC-yield than with the NAD<sup>+</sup>-GDH system at the cost of a slightly eroded enantioselectivity (Table 1, entries 1, 10). In combination with the NADP<sup>+</sup>-GDH system however, it outperformed both regeneration with mNAD<sup>+</sup> **3**-ATHase or with NAD<sup>+</sup>-GDH (Table 1, entry 2).<sup>14</sup> Regeneration of NADH or NADPH with the ATHase led to low GC-yield and nearly racemic product **7**, suggesting that the *N*-ethyl-2-methylmaleimide **6** is a better substrate than NAD(P)<sup>+</sup> for the ATHase (Table 1, entries 8, 9) leading to its direct (racemic) reduction by the {Ir-H}-moiety.









To alleviate the erosive effect of the direct substrate (**6**) reduction on enantioselectivity of the cascade process, the loading of mNAD<sup>+</sup> **3** was set to 10 mol% - resulting in an *e.e.* of 91-93% (see Table S4). Gratifyingly, a set of alternative ERs (from Codexis) also readily accepted mimic **3** and were tested in the cascade reaction. Except for ERED-P1-E01, none of these compared favorably with *TsOYE*, (Table S5).

Upon increasing the reaction time from four to seven hours, the initial loading of iridium could be reduced from the 0.1% to 0.05% without compromising the GC-yield (Table 1, entries 10, 11). Slow addition of the substrate allowed to improve the *e.e.* further (up to 95-98% *e.e.*) by minimizing the background direct reduction of substrate **6** (Table 1, entries 11, 12). A preparative scale reaction with *N*-ethyl-2-methylmaleimide **6** (1.0 mmol) resulted in > 99% GC-yield (61% isolated yield) and 98 % *e.e.* (Table 1, entry 13).

Ketoisophorone **8** and 2-methylcyclohexenone **9** were reduced with moderate to good GC-yields when applied to the cascade (Table 1, entries 14-19). The lower enantioselectivity for the reduction of **8** reflects the trend observed in reactions with stoichiometric quantities of cofactor and is partly due to a known non-enzymatic racemization of the reduced product over time.<sup>14,61</sup>

**Table 1. Selected results for ERs using mNAD<sup>+</sup> **3**.<sup>a</sup>**

Entry	ER	IrC-S112K [mol%]	(m)NAD(P) <sup>+</sup>	Substrate	Time [h]	<i>e.e.</i> [%]	GC-yield [%]	TTN <sub>Ir</sub>	TTN <sub>ER</sub> <sup>h</sup>	
1	<i>TsOYE</i>	GDH <sup>b</sup>	NAD <sup>+</sup>	<b>6</b>	4	>99 ( <i>R</i> )	65	195 <sup>g</sup>	118	
2	<i>TsOYE</i>	GDH <sup>b</sup>	NADP <sup>+</sup>	<b>6</b>	4	>99 ( <i>R</i> )	>99	>298 <sup>g</sup>	181	
3	–	0.1 (IrA) <sup>c</sup>	–	<b>6</b>	4	0	10	104	80	
4	–	0.1 (IrB) <sup>c</sup>	–	<b>6</b>	4	2 ( <i>R</i> )	5	5	4	
5	–	0.1 (IrC) <sup>c</sup>	–	<b>6</b>	4	0	10	96	74	
6	–	0.1	–		<b>6</b>	4	3 ( <i>S</i> )	14	136	105
7	<i>TsOYE</i>	0.1	–	<b>6</b>	4	4 ( <i>S</i> )	11	113	374	
8	<i>TsOYE</i>	0.1	NAD <sup>+</sup>	<b>6</b>	4	4 ( <i>R</i> )	12	117	390	
9	<i>TsOYE</i>	0.1	NADP <sup>+</sup>	<b>6</b>	4	6 ( <i>R</i> )	14	139	464	
10	<i>TsOYE</i>	0.1	mNAD <sup>+</sup> <b>3</b>	<b>6</b>	4	93 ( <i>R</i> )	85	853	2843	
11	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>	<b>6</b>	7	91 ( <i>R</i> )	87	1746	8730	

12	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>6</b>	7 <sup>d</sup>	95 (R)	>99	>1980	>9900
13	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>6</b>	7 <sup>e</sup>	98 (R)	>99 (61) <sup>f</sup>	>1980	>9900
14	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>8</b>	8	86 (R)	30	556	2980
15	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>8</b>	8 <sup>d</sup>	85 (R)	23	467	2333
16	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>8</b>	24	72 (R)	48	951	4756
17	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>9</b>	8	94 (R)	69	1385	6531
18	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>9</b>	8 <sup>d</sup>	94 (R)	69	1369	6457
19	<i>TsOYE</i>	0.05	mNAD <sup>+</sup> <b>3</b>		<b>9</b>	12	82 (R)	83	1655	7799

<sup>a</sup>Experiments were performed in 0.6 M MOPS, 2.0 M HCOONa (pH 7.0) at 30 °C and on a 400 µl scale (except entry 13); 10 mM (entries 1-10) or 30 mM (entries 11-19) substrate, 3 µM *TsOYE*, 1 mM (entry 10) or 3 mM (entries 11-19) mNAD<sup>+</sup> **3** or 1 mM NAD(P)<sup>+</sup> (entries 1, 2, 8, 9). Average values for triplicate reactions are shown (except for entry 13); standard deviations were ≤ 1.4%. GC-yields were determined by GC with the following internal standards: benzyl alcohol for **6**, cyclohexanol for ketoisophorone (**8**) and 2-methylcyclohexanone (**9**). Absolute configuration of **7** was tentatively assigned by comparison with *N*-phenyl-3-methylsuccinimide produced by *TsOYE* reduction.<sup>14</sup> The absolute configuration of reduced **8** (levodione **10**) by *TsOYE* reduction has been reported previously.<sup>14</sup> Absolute configuration of reduced **9** (2-methylcyclohexanone **11**) was tentatively assigned by optical rotation and comparison with literature data (see SI). <sup>b</sup> 1.2 mg/ml of glucose dehydrogenase (GDH-105 from Codexis) and 100 mM glucose were used instead of ATHase IrC-Sav S112K and HCOONa. <sup>c</sup> IrA, IrB, and IrC were used instead of IrC-S112K, respectively. <sup>d</sup> Half of the substrate was added at 0 h and after 1 h, respectively. <sup>e</sup> Preparative scale reaction, 141 mg of **6** was added to the reaction mixture in portions every 10 min for 170 min. <sup>f</sup> isolated yield. <sup>g</sup> An accurate turnover number for the commercial GDH can not be calculated, since the enzyme is only semipurified according to the manufacturer. We calculated an upper limit based on 78% purity (determined by the Bradford assay)<sup>62</sup> and a molecular weight of 28 kDa.<sup>63,64</sup> The reaction conditions have not been optimized for GDH. Alternative enzymes with potentially higher activity (e.g. Codexis CDX-901) are commercially available. <sup>h</sup> TTNs for *TsOYE* represent a lower limit based on protein content in the lyophilised preparation (20 wt%) as determined by the Bradford assay.

In summary, the first efficient regeneration system for NADH mimics has been developed and its applicability demonstrated in the combination with a range of ene reductases. Further research will focus on the evaluation in concurrent reactions with other enzymes that accept mimics such as monooxygenases.<sup>18</sup> To the best of our knowledge, the catalytic performance (i.e. TTN) of this Ir-based system for (m)NADH regeneration is the best reported for non-enzymatic regeneration systems. Potential regeneration systems for mNAD<sup>+</sup>s will have to be scrutinized with respect to achievable turnover frequency and total turnover number not only for the regenerating metal catalyst, but also for the mimic.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. The supporting information include follows; materials, instruments, buffers and stock solutions for the enzymatic reactions, experimental procedures, GC analysis conditions and their representative chromatogram, the comparison of catalytic activities of IrC and ATHase variants for mNAD<sup>+</sup> **1** reduction, the stability of mNAD<sup>+</sup> **1**, the influence of formate on the activity of the *TsOYE*, the numerical values for the fingerprint displayed in Scheme 2, the comparison of the reduction rates of mNAD<sup>+</sup> **1** and mNAD<sup>+</sup> **3** vs NAD(P)<sup>+</sup> by the ATHase variant S112K, the spectral changes of the reduction of mNAD<sup>+</sup> **3** to mNADH **3**, the influence of the iridium cofactor IrC and ATHase S112K on *TsOYE*, the optimization of reaction condition for the cascade reactions, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR of the *N*-ethyl-2-methylsuccine imide obtained in the preparative cascade reactions (PDF)

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### Author Contributions

FH, VK and TRW designed the project, YO and CEP designed and performed the experiments and analyzed the results. YO, VK and TRW wrote the manuscript.

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## ABBREVIATIONS

mNADH, NAD(P)H mimic; Sav, streptavidin; AME, artificial metalloenzyme; ATHase, artificial transfer hydrogenase; ER, ene reductase; *TsOYE*, the Old Yellow Enzyme family from *Thermus scotoductus*.

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