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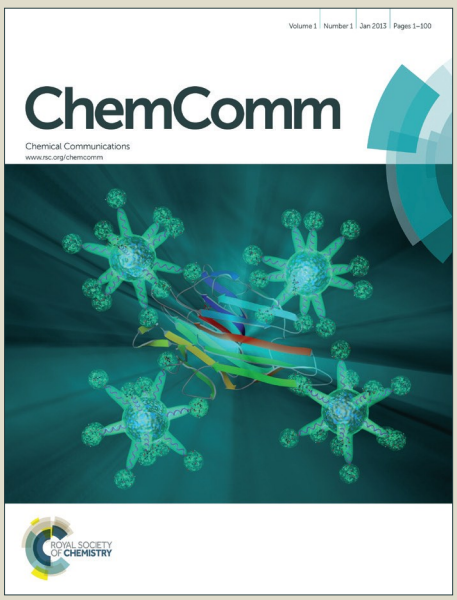

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New Artificial Fluoro-Cofactor of Hydride Transfer with Novel Fluorescence assay for Redox Biocatalysis

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New artificial fluoro-cofactor was developed for the replacement of natural cofactors NAD(P), exhibiting high hydride transfer ability. More importantly, we established a new and fast screening method for the evaluation of the properties of artificial cofactors based on the fluorescence assay and visible color change.

Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and their reduced form, NADH and NADPH are ubiquitous in all living because more than 400 oxidoreductases require NAD(P) as cofactors. Although there are several methods for in situ regeneration of NAD(P), including enzymatic,[1] chemical,[2] and electrochemical[3] regeneration, high costs and low-stability of NAD(P) urge people to find out artificial cofactors which can replace and even surpass NAD(P)[4]. NAD(P) contain two parts, the nicotinamide moiety acting as hydride donor or accepter and the adenine dinucleotide moiety playing an important role in separating between anabolic and catabolic pathways.[5] Although the anabolic and catabolic pathways are necessary for survival, it is not essential to realize hydride transfer in redox biocatalysis. Hence a number of nicotinamide-containing artificial cofactors have been reported (Fig. 1). In chemical catalysis, Hantzsch ester (HEH) acts as reductant in asymmetric hydrogenation of benzoxazinones successfully[6] and another artificial cofactor 9,10-dihydrophenanthridine (DHPD) has been designed for asymmetric hydrogenation of benzoxazinones, benzoxazines, quinoxalines and quinolones,

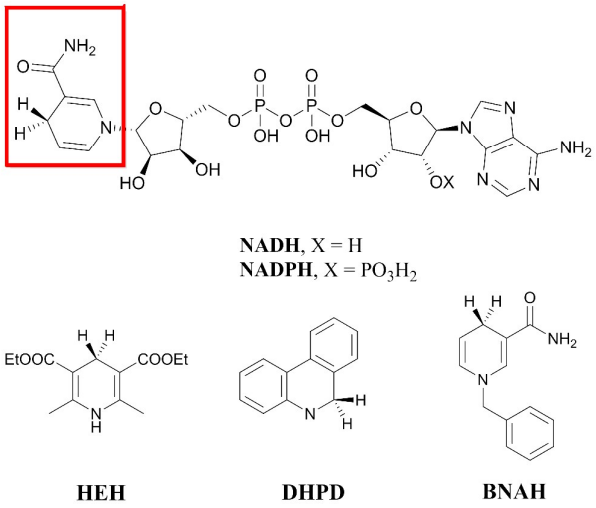

Figure 1 Structures of NAD(P)H and reported artificial cofactors

resulting in excellent activities and enantioselectivities.[7] Moreover, a widely-used artificial cofactor BNAH has been reported to react with oxidoreductase such as enoate reductases for C=C bioreduction,[8] and horse liver alcohol dehydrogenase for chiral synthesis.[9] Most notably, Nigel et al. reported the cocrystal structures of flavin-containing enzyme ene reductase in complex with NADH and representative artificial cofactor, validating that both natural and artificial cofactors shared similar π-π stacking effect and occupied the same region of the active sites.[10]

Inspired by these discoveries, here we reported novel artificial cofactors based on the 1,4-dihydropyridine skeleton. These artificial redox coenzymes are inexpensive to synthesize and stable enough to prolong the lifetime of enzymatic fuel cells[11] with lower potential than NADH. Due to the wide application of fluorine in drug discovery and development, we also introduced fluorine in our scaffold to expand the properties and synthetic methodologies which surprisingly produce a more facile access to a wide range of fluorinated

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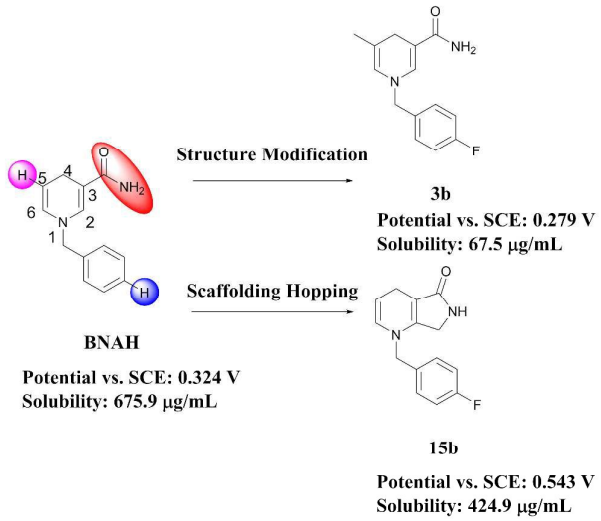
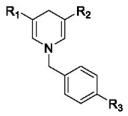


Figure 2 Diagram for the structural optimization strategy

artificial cofactors. These artificial cofactors allow the replacement of NAD(P)H to transfer the hydride to reductase despiting their apparently minimal structure to the native coenzymes. These artificial cofactors may be applied in sugar-powered biobattery, meanwhile boosting the development of artificial catalysts in asymmetric synthesis. Moreover, in view of complicated existing evaluation methodology of artificial cofactors, we chose a substrate that could give remarkable color change after specific reduction.

Table 1 Potential and solubility of biomimetic cofactors



Compound	R_1	R_2	R_3	E[a] potential(V) vs. SCE	Solubility[b] μg/mL
		NADH		0.551	$>10^4$
1(BNAH)	H	$CONH_2$	H	0.324	675.9
2b	CH_3	$CONH_2$	H	0.312	298.4
3b	CH_3	$CONH_2$	F	0.279	67.5
4b	CH_3	$CONH_2$	$OCH_2CH_2OCH_3$	0.348	705.5
5b	H	$CSNH_2$	H	0.451	89.1
6b	H	$CSNH_2$	F	0.425	<10
7b	H	$CSNH_2$	$OCH_2CH_2OCH_3$	0.508	100.5
8b	H	$COOCH_3$	H	0.420	115.8
9b	H	$COOCH_3$	F	0.363	32.9
10b	H	$COOCH_3$	$OCH_2CH_2OCH_3$	0.408	62.8
11b	CH_3	$COOCH_3$	H	0.349	30.9
12b	CH_3	$COOCH_3$	F	0.318	<10
13b	CH_3	$COOCH_3$	$OCH_2CH_2OCH_3$	0.336	100.2
14b			H	0.578	918.7
15b			F	0.543	424.9
16b			$OCH_2CH_2OCH_3$	0.563	964.9

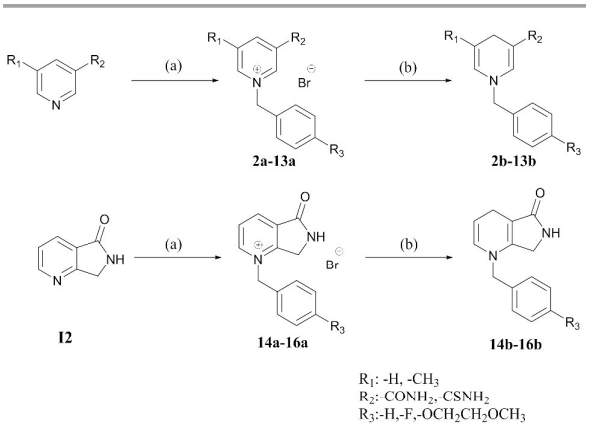
[a] Potential of aitificial cofactors and NADH recorded at a glassy carbon electrode. The voltage scan rate was 100 mV s^{-1}. [b]Solubility of biomimetic cofactors conducted by UV-visable spectrophotometer in 0.1 M PBS buffer, pH 7.4.

Each artificial cofactor has a unique potential, E, that indicates the ability of the compound to donate or accept electrons.[5] In order to lower its reducing potential, much focus has been emphasized on the structure modification and scaffold hopping of 1,4-dihydropyridine skeleton (Fig. 2).

First, we focused on the modification of the C-3 position with different substituents on the pyridine ring, whose position is the most important, owing to its role as coordinating center in the active catalytic pocket of reductase.[9] We replaced -$CONH_2$ with -$CSNH_2$ and -$COOCH_3$ in C-3 position of the 1,4-dihydropyridine skeleton because they have more tightness coordination ability with reductase.[12] In order to screen skeletons and further optimize hydride transfer ability of new artificial cofactors, we designed a five member lactam ring with immobilized intramolecular amide bond which could strengthen π-π stacking with reductase. Second, we attempted to introduce methyl on the C-5 position because methyl could inhibit protonation of the 5,6-double bond with less negative effect on hydride transfer ability to C4 (**8b** vs. **11b**) (**Table 1**).[13] Third, according to known literature,[14] 1-benzyl substituent on the nitrogen atom at the ring exerted a substantial electron-withdrawing effect that could favorably strengthen hydride transfer ability to C4. Hence, we introduced high electron-negativity group ether side chain and fluorine atom to facilitate transferring hydride. As a kind of conformational element in vivo, the introduction of F will lead an effect on benzyl conformation and influence the binding mode with the receptor.[15] Notably, the small size of fluorine may probably not disturb artificial cofactors entry into pockets.

The preparation of 1,4-dihydropyridine analogues started with commercially available substituted nicotinamide, methyl nicotinate, thionicotinamide and **I2**.[16] Through straightforward two steps, substituted nicotinamides were alkylated by benzyl bromide[17] in THF or CH_3CN to obtain bromide salts and reduced by $Na_2S_2O_4$ to yield 1,4-dihydropyridines (**2b-16b**) (Scheme 1).[18]

Substitution with F at the para- position of the 1-benzyl yielding **12b**, which displays lower potential than **11b** due to



Scheme 1 Reactions and conditions: (a) benzyl bromide, CH_3CN or THF reflux for 6 h; (b) $NaHCO_3$, $Na_2S_2O_4$, H_2O, in dark, Ar at rt for 3 h.

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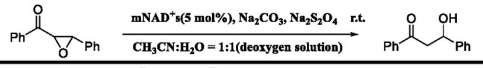
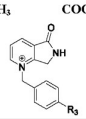
electron-withdrawing effect as we expected. (**3b** vs **2b**, **6b** vs **5b**, **9b** vs **8b**, **15b** vs **14b**) (Table 1, Fig. S1, ESI). Besides, sugar-powered biobatteries usually run on the aqueous buffer so we determine the water solubility of artificial cofactors. Though the introduction of F may sacrifice solubility to some extent, the solubility of **15b i**s just a bit lower than BNAH, without impeding the use of this artificial cofactors.

To appraise the hydride transfer ability of our artificial cofactors, we utilized the hydrogenation of α,β-epoxy ketones to β-hydroxy ketones that mediated through a catalytic amount of artificial cofactors in chemical system to evaluate the hydride transfer ability of our artificial cofactors.[19] In this enzyme-free reaction, $Na_2S_2O_4$ was used as the reducing agent to regenerate BNAH from BNA^+Br^-, with H_2O as hydride source, and the best optimized condition is CH_3CN/H_2O (1:1, v/v) at 25 °C, giving complete conversion with high isolated yields.

As shown in the Table 2, all artificial cofactors could transfer hydride to form β-hydroxy ketones, and the isolated yields of the best promising compounds such as **9b** and **12b** are up to 85%, higher than positive control BNAH (63%, respectively) which is outperform than natural coenzymes through steady-state-kinetics.[10] The reaction rates of compounds **5b**-**7b** are faster than other compounds, strongly suggesting better σ-donor S in this position could shorten reaction time indeed. We were pleased to find that all artificial cofactors could realize hydrogenation of α,β-epoxy ketones to form β-hydroxy ketones in this optimized reaction conditions, which means that our artificial cofactors could act as hydride donor in this chemistry system. Besides, among these five series cofactors, fluoro-cofactors displayed higher isolated yields than the cofactors substituted by H and $-OCH_2CH_2OCH_3$.

Table 2 Evaluation of artificial cofactors through chemistry method



compound	mNAD⁺s			time（h）	yield[a]
	R_1	R_2	R_3		
1(BNAH)	H	$CONH_2$	H	30	63%
2b	CH_3	$CONH_2$	H	30	65%
3b	CH_3	$CONH_2$	F	30	81%
4b	CH_3	$CONH_2$	$OCH_2CH_2OCH_3$	30	70%
5b	H	$SONH_2$	H	24	74%
6b	H	$SONH_2$	F	24	83%
7b	H	$SONH_2$	$OCH_2CH_2OCH_3$	22	75%
8b	H	$COOCH_3$	H	26	74%
9b	H	$COOCH_3$	F	27	85%
10b	H	$COOCH_3$	$OCH_2CH_2OCH_3$	26	83%
11b	CH_3	$COOCH_3$	H	26	72%
12b	CH_3	$COOCH_3$	F	26	85%
13b	CH_3	$COOCH_3$	$OCH_2CH_2OCH_3$	24	81%
14b			H	48	73%
15b			F	48	80%
16b			$OCH_2CH_2OCH_3$	48	78%

[a] Isolated yield through Flash Column Chromatography. Reactions were conducted in deoxygenated CH_3CN:H_2O (v/v, 1:1) at room temperature.

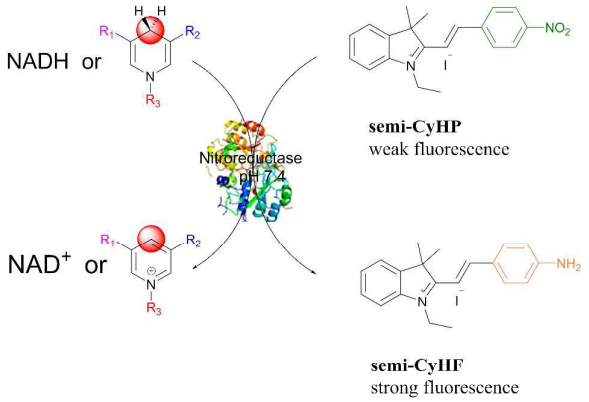


Figure 3 Reaction mechanism of synthetic artificial cofactors

These phenomena were in consistent with the results of cyclic voltammetry, indicating the advantage of the introduction of fluorine.

Furthermore, we designed a new assay to evaluate artificial cofactors that could react with reductase. This new assay was better than current assays, such as UV-visible absorption spectra, GC analyses, HPLC, steady-state kinetics. We selected a flavin-containing enzyme nitroreductase (NTR) from *Escherichia coli* and a fluorescence quenched substrate that will give fluorescence signals observed by naked eyes if reduced. Through the optical signal, we can judge whether the artificial cofactor can react with nitroreductase for the hydride transfer (Fig. 3).

NTR could effectively reduce nitroaromatic compounds to the corresponding amines in the presence of NADH as an electron donor by transferring the hydride. NTR exhibits equal capability of using either NADH or NADPH as a cofactor. Richard et al. demonstrated that the adenine, dinucleotide moiety, was not necessary and NTR could recognize simple 1,4-dihydropyridine compounds as effective as NAD(P)H in its ability to transfer hydride.[20]

Our group previously has reported semi-CyHP could be used as a selective off-on fluorescent probe which could detect NTR. Until reduced by NADH catalyzed by NTR, the amino group of semi-CyHF reconstructed the electronic push–pull system and a strong fluorescence was observed.[21] Without NADH or replacing NADH with other biological reductants such as glutathione (GSH), homocysteine (Hcy), dithiothreitol (DTT), cysteine (Cys), remarkable enhancement couldnot be obtained. This result demonstrated the importance of NADH in this system for transferring the hydride to NTR. These results suggested that fluorescence spectrosopy response could be used to evaluate the effects of our artificial cofactors.

The assay of artificial cofactors toward the reduction of semi-CyHP was performed in the phosphate buffered saline (PBS) buffer at 37 °C. The fluorescence of semi-CyHP solution (10^{-5} M) was undetectable when excited at around 490 nm. After addition of 2.5 μg mL^{-1} of NTR and 5×10^{-4} M NADH, a strong fluorescence enhancement at around 575 nm was observed. The reduction of the probe semi-CyHP was realized

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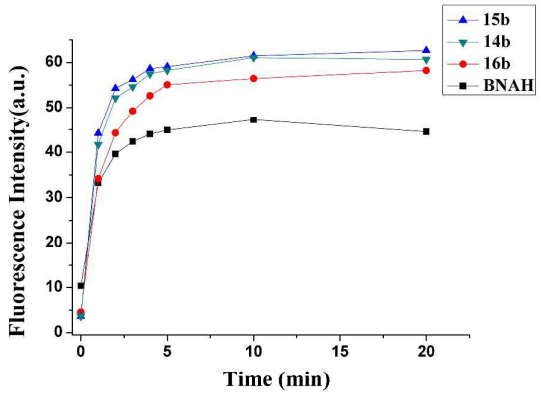

Figure 4 Fluorescence response of probe semi-CyHP (10^{-5} M) after adding artificial cofactors (5×10^{-4} M) and NTR (2.5 μg mL^{-1}) in 0.1 M PBS buffer (pH 7.4) with 1% (v/v) DMSO at 37 °C. The fluorescent intensity data were collected after certain time intervals at around 575 nm as indicated in the figure with excitation at 490 nm. Silt: 10, 10 nm.

and the reductive product semi-CyHF was formed as expected. By utilizing this three-component biocatalysis system which selectively responds to NADH, we could replace NADH with our artificial cofactors (**2b-16b**) (Fig. S2, ESI) to appraise the effects of artificial cofactors based on the fluorescence enhancement. The widely-used artificial cofactor BNAH could increase fluorescence intensity by 5-fold. But 12-fold enhancement in fluorescence emission at 575 nm was observed by incubating semi-CyHP with **14b, 15b**, **16b** and NTR (Fig. 4). This result could be ascribed to the enhanced π-π stacking effect of pyrido dihydropyrrolo scaffold (**14b, 15b**, **16b)** than BNAH. These results strongly suggest that an appropriate, convenient, visible and high resolution evaluation system has been established to appraise the hydride transfer ability of artificial cofactors. These pyrido dihydropyrrolo analogues could combine more tightly with NTR so that could realize the reduction of nitro group in semi-CyHP, inducing the change of color noticed by naked eyes and 12-fold enhancement of fluorescence intensity.

In summary, through rational design, we developed novel class artificial cofactors with low potential and good solubility. Moreover, we have established valid evaluation method based on fluorescence sensor to evaluate the hydride transfer ability when co-working with flavin-containing enzyme. This novel assessment system, to the best of our knowledge, was found to be the first time. These results prove that **14b, 15b, 16b** perform better hydride transfer ability than widely-used artificial cofactor BNAH. The introduction of fluorine into pyrido dihydropyrrolo analogues results in **15b**, featuring several advantages, lower reduction potential than NADH, high isolated yield in chemical system and 12-fold enhancement of fluorescence intensity in biocatalysis.

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