Biomimetic NAD^+ Models for Tandem Co-Factor Regeneration, Horse Liver Alcohol Dehydrogenase Recognition of the 1,4-NADH Derivatives, and Chiral Synthesis [*][**]

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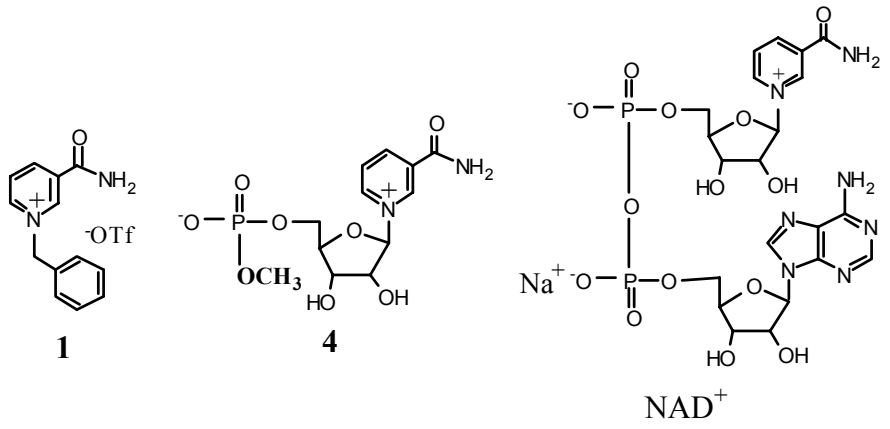
The biocatalysis discipline has the potential to generate important chiral organic compounds by the use of enzymes, usually in the presence of critical co-factors.[1,2] Therefore, practical methods for the regeneration of the co-enzyme, 1,4-NADH, have continued to be of significance in the biocatalysis field.[3-5] In this vain, a variety of transition metal hydrides have been evaluated as catalysts for the regioselective reduction of NAD^+ and NAD^+ models to the corresponding 1,4-NADH derivatives in order to attempt to develop faster rates and a more economical regeneration process.[6-9] In the most significant example, Steckhan and co-workers have described the use of *in situ* generated $[Cp^*Rh(bpy)(H)]^+$ ($Cp^* = \eta^5\text{-}C_5Me_5$, bpy = 2,2'-bipyridyl), for the regiospecific reduction of natural NAD^+ to 1,4-NADH,[7] and then demonstrated the co-factor regeneration process in

horse liver alcohol dehydrogenase enzymatic, chiral reduction reactions.[10-12]

More importantly, we recently reported on the source of this unusually high regioselectivity for 1,4-NADH and other mechanistic aspects with a model NAD^+ compound, 1-benzylnicotinamide triflate, **1**, in 1:1 H_2O/THF using $[Cp^*Rh(bpy)(H_2O)](OTf)_2$, **2**, as the catalyst precursor, and sodium formate as the hydride source to exclusively provide the kinetic product, 1-benzyl-1,4-dihydronicotinamide, **3**.[13] In addition, we also recently utilized an aqueous NAD^+ model, β-nicotinamide-5'-ribose methyl phosphate, **4**, and demonstrated its similar regioselective reduction with *in situ* formed $[Cp^*Rh(bpy)(H)]^+$ to the corresponding 1,4-dihydronicotinamide-5'-ribose methyl phosphate, **5**, at pH 6.5.[14] It is important to note that NAD^+ model, **4**, has some structural similarities to NAD^+, with particular emphasis on the mono-ribose phosphate moiety, but with no pyrophosphate nor adenosine substituents, *while also recognizing that the structure of the NAD^+ biomimic, **1**, has a simple 1-benzyl group in place of the ribose, pyrophosphate, and adenosine groups*.



The initial rates (r_i) of the regioselective reduction of both **1** in 4:1 H_2O/THF and **4** in H_2O at pH 6.5, with *in situ* generated $[Cp^*Rh(bpy)(H)]^+$, to their corresponding 1,4-dihydro analogs, **3** and **5**, were comparable to NAD^+ in H_2O at pH 6.5 (turnover frequency [TOF], 20 h^{-1}).[14] Therefore, we decided to attempt to use both **1** and **4** as biomimics of NAD^+ in chiral reduction reactions, in conjunction with the above-mentioned co-factor regeneration method.[13,14] Moreover, recent studies with other NAD^+ biomimics by Lowe and co-workers showed that the oxidation of primary alcohols to aldehydes occurred, along with 1,4-NADH biomimic production, *but the reverse reaction, i.e., the reduction of aldehydes to alcohols, along with NAD^+ biomimic production, was not observed.*[15-17]

Thus, the pertinent question we asked ourselves was: what is the role of each substituent on the

1,4-dihydronicotinamide nucleus, including ribose, pyrophosphate, and adenosine groups, and will HLADH enzymatic recognition at the binding site for the co-factor prevail to provide chiral reduction products with the most important structural feature still present in our biomimetic 1,4-NADH models; namely, the 1,4-dihydronicotinamide nucleus?[18-21]

The preliminary, tandem co-factor regeneration and chiral synthesis experiments with substrates **6-10** (Chart) and NAD^+ biomimics, **1** and **4**, with NAD^+ as a comparison, provided quite startling and dramatic results in that; for example, the aryl substituted, prochiral ketone, $PhCH_2CH_2C(O)CH_3$, **6**, in the presence of HLADH, was reduced to give the corresponding alcohol, $PhCH_2CH_2CH(OH)CH_3$, with >93 % ee (S-enantiomer) and a TOF of ~30 d^{-1}; both NAD^+ models, **1** and **4**, gave similar chiral synthesis results compared to NAD^+ (Table 1). Clearly, all that is apparently necessary for HLADH site recognition of the 1, 4-NADH biomimics, **3** and **5**, is the 1,4-dihydronicotinamde moiety, while the 1-benzyl and ribose-5'-methyl phosphate role in possibly binding in the hydrophobic pocket of HLADH does not appear to compromise the transfer of hydride to the ketone substrate to provide chiral alcohol (Scheme 1)!

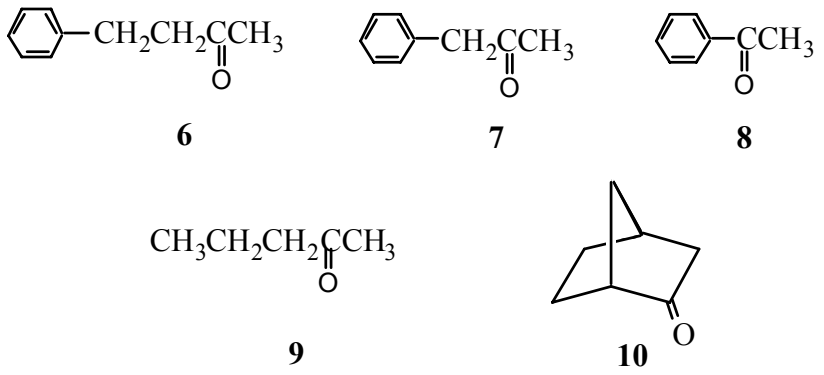
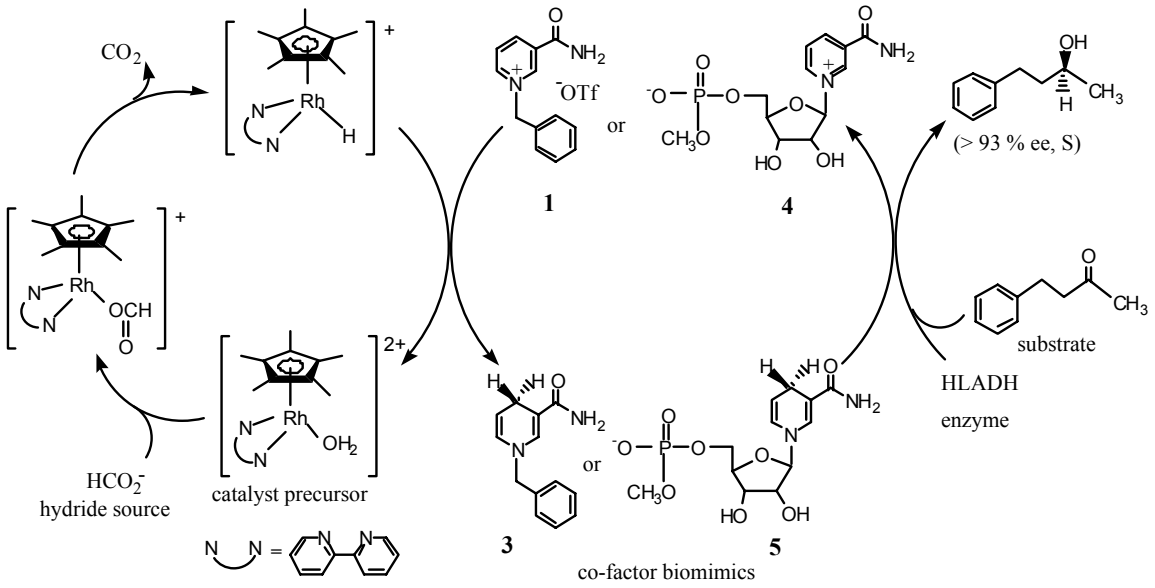


Chart: Prochiral Ketone Substrates



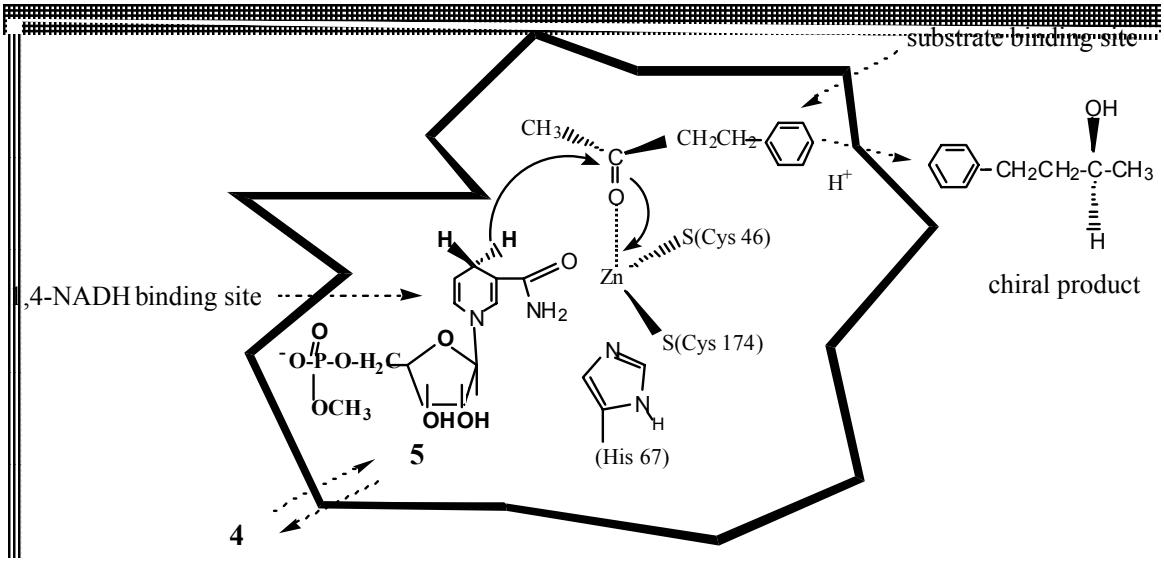
Scheme 1: Plausible catalytic reduction cycle for the synthesis of 1,4-NADH biomimics, **3** or **5**, followed by a catalytic cycle for chiral alcohol synthesis from ketone substrates with the enzyme, HLADH.

A structure-reactivity study (**Chart**) of a variety of ketone substrates (**Table 1**) further showed that benzylmethyl ketone, $PhCH_2C(O)CH_3$, **7**, also provided the corresponding chiral alcohol, $PhCH_2CH(OH)CH_3$ (>99 % ee, *S*-enantiomer, TOF, 18 d^{-1}), while benzophenone, $PhC(O)CH_3$, **8**, was extremely slow (TOF, 2 d^{-1}) in providing chiral product, $PhCH(OH)CH_3$, after 24 h, but the final result was still >93 % ee for the S-enantiomer. The alkyl ketone, **9**, also was studied, and provided an 85 % ee for the predominate S-alcohol, $CH_3CH_2CH_2CH(OH)CH_3$, which was similar to NAD^+, and may demonstrate that the binding site for this type of alkyl ketone is not as rigid as the phenethyl ketone binding site to engender higher chirality to the alcohol product.

Alternatively, the bulky, aliphatic ketone, norcamphor, **10**, which is actually a commercial mixture of two enantiomers, reacted at not only different reduction rates, but also provided a different diastereomeric mixture of predominantly *endo* chiral alcohols. As shown in **Table 2**, one enantiomer of **10** gave an approximately equal mixture of the *exo* and *endo* alcohols, while the other gave exclusively the *endo*-alcohol with no *exo*-alcohol detected.

Interestingly, the hydride transfer pathway from 1,4-NADH to the HLADH bound ketone is still very controversial, with various mechanisms proposed, but none

appear very satisfactory.[22] The previously formulated results indicated that the initial binding process of natural
1,4-NADH occurs in proximity to the Zn^{2+} metal ion center of HLADH, and the Zn^{2+} bound substrate.[23,24] In a manner similar to that of the $[Cp^*Rh(bpy)H]^+$ binding of biomimic **1** for regioselective production of **3**,[13,14] we also envision the possibility that the carbonyl of the amide group of 1,4-NADH, and that of biomimics, **3** or **5**, might weakly bind to the Zn^{2+} metal ion center to stabilize this hydride transfer process. This possible Zn^{2+} binding process of co-factor during the transfer of hydride from **5** to the *re* face of the carbonyl group could also conceivably add stability to the microscopic reverse reaction; i.e., hydride transfer from a specific C-H of RCH_2OZn to **4**, to give a C=O product and **5**. We have in fact shown that the reverse reaction, *S*-$CH^-{}_3CH_2CH_2CH(OH)CH_3$ to
$CH_3CH_2CH_2C(O)CH_3$, **9**, occurs with NAD^+ biomimic, **4**, while the *R*-enantiomer was found to be extremely slow.



Scheme 2: Postulated active site model for HLADH with 1,4-NADH biomimic, **5**, as hydride source and phenethylmethyl ketone, **8**, as substrate.

This potential binding regime allows the Zn^{2+} metal center to become 4 or 5 coordinate during the 1,4-NADH hydride transfer process in the presence of the carbonyl substrate. Recently, Sagi and co-workers[28] have strengthened the concept of the Zn^{2+} metal ion center being a template for much of the ADH reactivity. They demonstrated with a thermophilic ADH enzyme, TbADH, that the Zn^{2+} metal ion center exhibits a pentacoordinate structure, and developed an active site model that reflected their results with Asp_{150},His_{59},Glu_{60},Cys_{37}, and DMSO as the surrogate substrate, coordinated to the Zn^{2+}.[25]

In conclusion, we have demonstrated that biomimetic NAD^+ models, structurally modified to retain the nicotinamide nucleus and either one similar substituent, the ribose-5′-phosphate group, *or none*, the 1-benzyl group, compared to NAD^+, can be regioselectively converted to their 1,4-dihydronicotinamide analogs, and indeed, we have clearly shown for the first time, as far as we can understand, that these 1,4-NADH biomimics are recognized by the HLADH co-factor binding site for chiral synthesis of aryl/alkyl substituted alcohols

While the economic benefits of this discovery can not be fully evaluated at this time for biocatalytic applications, it is obvious from the presented results, and previous observations,[15-17] that some structural features of 1,4-NADH need not be present to afford enzyme recognition and chiral organic compound synthesis. We hope our results pave the way for the possible utilization of NAD^+ models, such as **1** and **4**, in a variety of biocatalytic processes of industrial importance; model **1** and other potential analogs especially being more stable under conditions that might cause the NAD^+ to be hydrolytically compromised.[26,27]

[*] Prof. R. H. Fish and Dr. H. C. Lo. Correspondence to RHF at rhfish@lbl.gov. We dedicate this manuscript to the

memory of Dr. E. Steckhan, a pioneer in tandem co-factor regeneration and chiral synthesis.

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Table 1: Enzymatic Reductions of Prochiral Ketones with NAD^+ Models **1** and **4:** Turnover Frequencies and Enantiomeric Excess[a,b]

substrate	product	% yield	TOF (d^{-1})	ee (%, S)
6	Structure here	90(91)	30(31)	93(93)
7	Structure here	55(59)	18(19)	>99(99)
8	Structure here	5(5)	4(4)	>96 (96)
9	Structure here	41(59)	14(20)	85(85)[c]

[a]Models **1** and **4** provided similar results within experimental error (< 2%). The results with NAD^+ are given in parenthesis. [b]The enantiomeric excess was determined by GC analysis with a modified β-cyclodextrin capillary column. [c]Based on the analysis of carbamate diastereomers with an optically pure isocyanate derivative.

Table 2: Enzymatic Conversion of Racemic Norcamphor: Turnover Frequency (TOF) and Yield using NAD Models **1** and **4**[a]

Substrate	Product		% Yield	TOF (d^{-1})
Structure here	Structure here	Sttructure here		
(+)-(1S, 4R)	(+)-(1S, 2R, 4R) 0.45 [0.48]	(+)-(1S, 2R, 4R) 0.0 [0.0]		
			86[94]	28 [29]
Structure here	Structure here	Structure here		
(-)-(1R, 4S) 0.5 equiv each	(-)-(1R, 2S, 4S) 0.21 [0.25]	(-)-(1R, 2R, 4S) 0.20 [0.21]		
10				

[a]Comparative [NAD^+] results

Experimental Section.

General Procedure for Biomimetic Reductions of Carbonyl Substrates: NAD^+ or NAD^+ Models, 1 and 4, as the Cofactors

$[Cp^*Rh(bpy)(H_2O)]OTf_2$ (1.9 mg, 2.61 x 10^{-3} mmol), sodium formate (17.8 mg, 261.69 x 10^{-3} mmol), NAD^+ (or NAD^+ models **1** or **4**) (8.1 mg, 11.23 x 10^{-3} mmol), and HLADH (10 units) were placed in a 10 mL Schlenk flask, and Schlenk techniques were followed to deoxygenate the solid mixture. Under positive argon pressure, 5 mL of potassium phosphate buffer (100 mM, pH = 7.02, de-O_2) and carbonyl substrate (83.58 x 10^{-3} mmol, de-O_2) were added via syringe, respectively. The reaction flask was immediately capped securely with a glass-stopper and shaken with a shaker in a 30°C water-bath. The reaction was monitored by GC, equipped with a β-cyclodextrin column (Supelco, β-DEX-225), and the products were identified by comparing the retention time with the corresponding chiral authentic samples, and were confirmed by GC-MS. The enantiomeric excess of the products was directly determined by calculating their relative areas from the chromatograms. The progress of the reaction was obtained by mixing the reaction aliquot with an internal standard (cyclohexanol in phosphate buffer), and then calibrating the measured relative area in the chromatogram with their corresponding response factors. The reaction was monitored for 24 h.

Control Experiments

No chiral alcohol product was formed without the presence of the HLADH enzyme, while no ketone was reduced to alcohol in the presence of the model cofactors, **1** and **4**. The latter result shows that **1** and **4** preferentially bind to the Cp*Rh metal ion center, in the presence of the ketone, and they are regioselectively reduced to their 1,4-NADH derivatives, **3** and **5**, all in the absence of HLADH.

General Procedure for Biomimetic Oxidation of S-2-Pentanol: NAD^+ and the Biomimic,4, as the Cofactors:

NAD^+ (83.58×10^{-3} mmol){ (or NAD^+ models **1** or **4**)} and HLADH (10 units) were placed in a 10 mL Schlenk flask, and Schlenk techniques were followed to deoxygenate the solid mixture. Under positive argon pressure, 5 mL of potassium phosphate buffer (100 mM, pH = 7.04, de-O_2) and *S*-2-pentanol (83.58×10^{-3} mmol) were added via syringe, respectively. The reaction flask was immediately capped securely with a glass-stopper and shaken with a shaker at room temperature. The progress of the reaction was monitored by GC, and the product ketone, 2-pentanone, was identified by comparing the retention time with that of an authentic sample. Under the same conditions, the oxidation of R-2-pentanol was tested as well, and its reaction rate was found to be much slower relative to that of *S*-2-pentanol. The relative rates of *S*- and *R*-2-pentanols in the

first 24 hr were ~ 4:1. Additionally, the reactions were found to reach an equilibrium after ~ 60 h (in the case of *S*-2-pentanol), where both 2-pentanol and 2-pentnone were present in the reaction mixture in a ratio of ~ 40 to 60. The same procedure described in the NAD^+ example was followed with the water soluble model **4**. Both racemic 2-pentanol and S-2-pentanol were tested, and similar results were obtained as stated in the NAD^+ case except that the reaction rate became slower after 24 h.