Supplementary data

Synthetic biomimetic coenzymes and alcohol dehydrogenases for asymmetric catalysis

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1. GENERAL MATERIALS AND METHODS

All commercially available reagents and solvents were purchased with the highest purity available and used as received. The horse liver alcohol dehydrogenase (HLADH) was purchased from Evocatal GmbH as ADH 210 HLADH (≥0.5 U/mg, protein concentration 0.22 mg/mg) and from Sigma-Aldrich as alcohol dehydrogenase equine (recombinant, expressed in *E. coli*, ≥0.5 U/mg). When referring to the 'crude' preparation of HLADH, the enzyme was used as received. When referring to the 'dialyzed' HLADH, the enzyme was further purified through dialysis. The activity of both the crude and dialyzed HLADH was measured by UV spectroscopy before use. 1-Benzyl-1,4-dihydronicotinamide and other analogues were synthesized as previously described.[1-2]

UV spectroscopy measurements were made using an Agilent Cary 60 UV-Vis spectrophotometer at the designated wavelength.

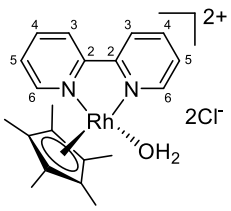
Nuclear magnetic resonance (NMR) spectra were recorded on an Agilent Technologies spectrometer at 400 MHz (^{1}H). Chemical shifts (δ) are reported in parts per million (ppm) relative to Me_4Si (δ 0 ppm) using the solvent residual signals of deuterated solvent (CD_3OD or $CDCl_3$). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet), coupling constant, integration, and assignment.

All gas chromatography (GC) analyses were carried out on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector (FID). More details are described in section 4, GC analyses p. S7.

2. EXPERIMENTAL PROCEDURES

2.1. Syntheses of the rhodium complexes

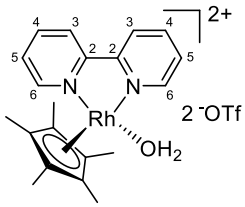
Pentamethyl cyclopentadienyl (2,2'-bipyridyl) rhodium(III) chloride [Cp*Rh(bpy)(H_2O)]Cl_2



The following method was adapted from a previous procedure.[3] 2,2'-Bipyridine (50 mg, 0.32 mmol) was added to a suspension of pentamethylcyclopentadienylrhodium(III) chloride dimer ([Cp*$RhCl_2$]$_2$ (97 mg, 0.16 mmol) in methanol (5 mL), and the orange solution was stirred at room temperature for 20 min. The product was precipitated with the addition of diethyl ether, then filtered and dried under vacuum to give [Cp*Rh(bpy)(H_2O)]Cl_2 as an orange solid (100 mg, 0.21 mmol, 65%). ^{1}H NMR (400 MHz, D_2O) δ 8.96 (d, *J* = 5.2 Hz, 2H, bpy H-6), 8.40 (d, *J* = 8.0 Hz, 2H, bpy H-3), 8.23 (td, *J* = 7.9, 1.3 Hz, 2H, bpy H-4), 7.83 (ddd, *J* = 7.4, 5.6, 1.6 Hz, 2H, bpy H-5), 1.67 (s, 15H, Cp); ^{13}C NMR (100 MHz, D_2O) δ 154.2 (bpy C-2), 151.6 (bpy C-6), 140.5 (bpy C-4), 128.5 (bpy C-5), 123.7 (bpy C-3), 98.0 (Cp), 8.0 (Cp-Me).

Pentamethyl cyclopentadienyl (2,2'-bipyridyl) rhodium(III) triflate [Cp*Rh(bpy)(H_2O)](OTf)$_2$

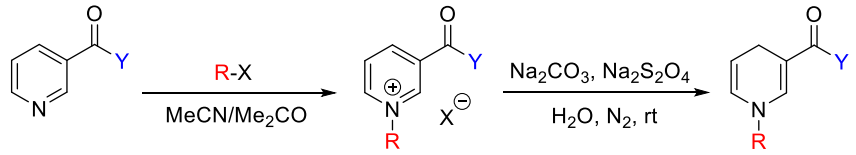
Silver trifluoromethanesulfonate (59 mg, 0.23 mmol) in water (1.5 mL, deoxygenated) was added to a



solution of [Cp*Rh(bpy)(H_2O)]Cl_2 (49 mg, 0.10 mmol) in water (4 mL, deoxygenated). The resulting suspension was stirred for 45 min at room temperature. The precipitate formed was filtered and washed with cold distilled water and the filtrate was lyophilized overnight to give a yellow solid (71 mg, 0.10 mmol, 98%). ^{1}H NMR (400 MHz, D_2O) δ ^{1}H NMR (400 MHz, D_2O) δ 9.15 (d, *J* = 5.2 Hz, 2H, bpy H-6), 8.50 (d, *J* = 8.0 Hz, 2H, bpy H-3), 8.35 (td, *J* = 7.9, 1.5 Hz, 2H, bpy H-4), 7.94 (t, *J* = 6.6 Hz, 2H, bpy H-5), 1.73 (s, 15H, Cp); ^{13}C NMR (100 MHz, D_2O) δ 155.0 (bpy C-2), 151.5 (bpy C-6), 141.5 (bpy C-4), 128.9 (bpy C-5), 124.1 (bpy C-3), 119.6 (q, *J* = 316 Hz, OTf), 97.9 (Cp), 7.9 (Cp-Me).

2.2. Synthesis of the nicotinamide coenzyme biomimetics

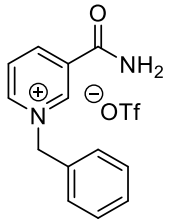
All the NCBs were synthesized as previously described and were readily available in our laboratory (Scheme 1).[1,4]



Y = NH_2, CH_3
X = Cl, Br, I
R = CH_2Ph, CH_3, $(CH_2)_3CH_3$, $(CH_2)_4SO_3^-$, $(CH_2)_2OH$, CH_2OH, CH_2CONH_2, $(CH_2)_2CONH_2$

Scheme S1. General synthetic route for the production of oxidized and reduced NCBs.

1-Benzyl-3-carbamoylpyridinium triflate BNA$^+$ $^-$OTf



Silver trifluoromethanesulfonate (175 mg, 0.68 mmol) was added to a solution of 1-benzyl-3-carbamoylpyridinium bromide (199 mg, 0.68 mmol) in methanol (5 mL). After 20 min, the white precipitate formed was filtered and the filtrate was concentrated under reduced pressure. The crude product was dissolved in a mixture of acetone/CH_2Cl_2 (1:1) and precipitated with the slow addition of diethyl ether to afford a pure white solid (247 mg, quantitative). ^{1}H NMR (400 MHz, D_2O) δ 9.34 (s, 1H), 9.04 (d, *J* = 6.2 Hz, 1H), 8.88 (d, *J* = 8.0 Hz, 1H), 8.16 (t, *J* = 7.2 Hz, 1H), 7.49 (s, 5H), 5.88 (s, 2H); ^{13}C NMR (100 MHz, D_2O) δ 165.7, 146.5, 144.3, 134.1, 132.2, 130.1, 129.6, 129.2, 128.5, 119.6 (q, *J* 310, OTf), 65.1.

The following NCBs were available and used (Figure S1):

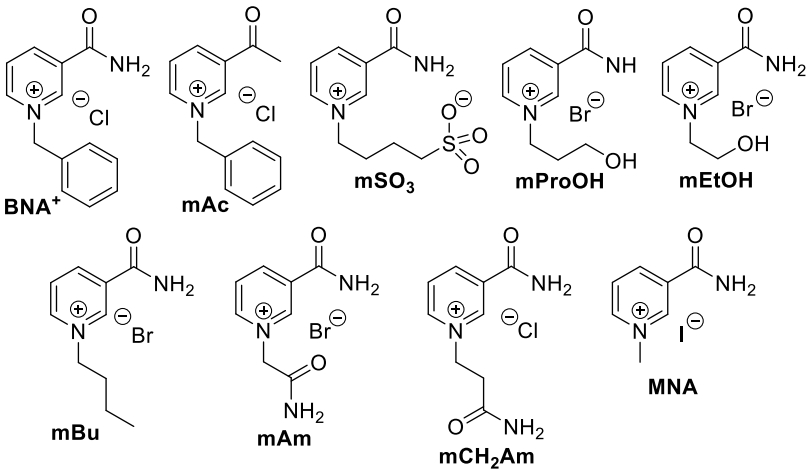


Figure S1. Oxidized synthetic analogues used in the studies.

- Oxidized forms:

BNA 1-Benzyl-3-carbamoylpyridinium chloride, ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.55 (m, 1H), 9.23–9.15 (m, 1H), 9.04–8.95 (m, 1H), 8.23 (m, 6.0 Hz, 1H), 7.68–7.33 (m, 5H), 5.95 (s, 2H);
mAc 1-benzyl-3-cacetylpyridinium chloride;
mSO$_3$ 1-(4-sulfonatobutyl)-3-carbamoylpyridinium;
mProOH 1-(3-hydroxypropyl)-3-carbamoylpyridinium bromide;
mEtOH 1-(2-hydroxyethyl)-3-carbamoylpyridinium bromide;
mBu 1-butyl-3-carbamoylpyridinium bromide, ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.53 (s, 1H), 9.22 (m, 1H), 9.00 (m, 1H), 8.26 (m, 1H), 4.75 (m, 2H), 2.13–1.99 (m, 2H), 1.47 (m, 2H), 1.02 (t, 3H);
mAm 1-(carbamoylmethyl)-3-carbamoylpyridinium chloride;
mCH$_2$Am 1-(2-carbamoylethyl)-3-carbamoylpyridinium chloride;
MNA 1-methyl-3-carbamoylpyridinium iodide.

- Reduced forms:
mBuH 1-butyl-1,4-dihydronicotinamide;
BNAH 1-benzyl-1,4-dihydronicotinamide.

A redox potential for BNAH is reported at E_0 = -361 mV, compared to the -320 mV of NADH, resulting in a modest overall ΔE(NAD/BNA) of +41 mV.

2.3. Dialysis of HLADH

The commercially purchased HLADH, a beige lyophilized powder, was dialyzed with a Dialysis Tubing Cellulose Membrane from Sigma-Aldrich (avg. diam. 6 mm, avg. flat width 10 mm, molecular weight cut-off 14,000 Da). 60 mg of the crude enzyme preparation were dissolved in 2 mL of potassium phosphate buffer (100 mM, pH 7.0) and pipetted into the cellulose membrane, which was submerged in 500 mL of the phosphate buffer. The solution was gently stirred in a cold room (4 °C) or ice bath for 6 h, changing the buffer every 1.5 h, and stored in the fridge overnight. The buffer was then changed to 10 mM potassium phosphate buffer (pH 7.0), stirred for another 30 min and the enzyme solution was lyophilized to give a white powder. The enzyme activity on benzaldehyde was measured with a UV assay and gave 0.62 U/mg. SDS-PAGE of the dialyzed HLADH showed the presence of other high molecular weight proteins, with the molecular mass of HLADH as the major one.

2.4. HLADH UV assays

The activity of HLADH was determined by UV assays on benzaldehyde. The change in absorbance of NADH was monitored at 340 nm under the following conditions:

100 mM potassium phosphate buffer (pH 7.0), 100 mM benzaldehyde, 10 mM NADH, 1 mg/mL HLADH. The buffer, benzaldehyde and NADH were mixed in a cuvette and incubated at 30 °C for 5 min. The enzyme solution was then added and change in absorbance was measured at 30 °C and 340 nm. The linear change in absorbance within the first minute of the measurement showed an enzyme activity of 0.70 U/mg for HLADH bought from Sigma Aldrich and 0.73 U/mg for HLADH from Evocatal GmbH evo-1.1.210 (protein concentration of 0.22 mg/mg).

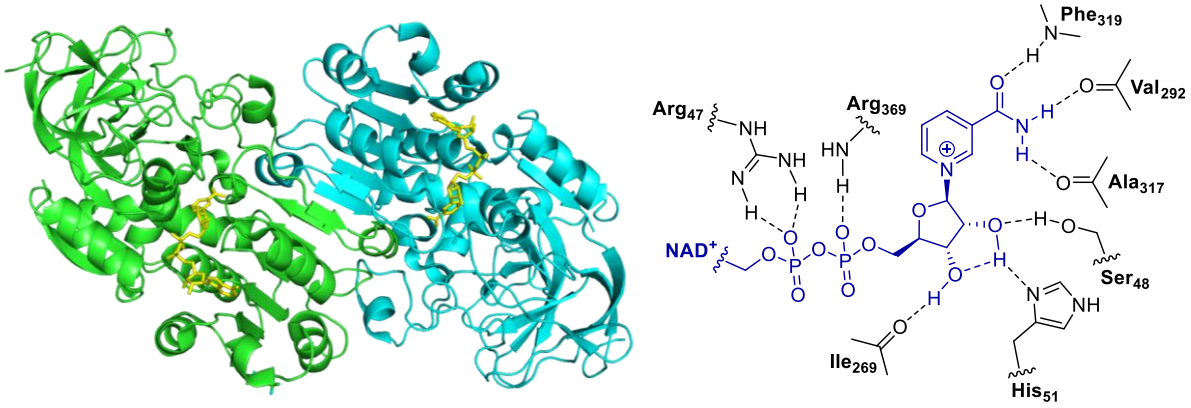


Figure S2. Left: Structure of HLADH complexed with NAD.[5] Right: Simplified schematic representation of the NAD (in blue) binding within the active site of HLADH (in black, adapted).[6-7]

2.5. ADH-A preparation

2.5.1. Bacterial strains and plasmids

The expression construct, pET15b-ADH-A, was transformed into chemically competent *E. coli* BL21(DE3). 50 µL competent cells and approximately 100 ng plasmid used. Transformed cells were grown for 1 h at 37 °C in LB without antibiotics. 100 µL were plated out on a LB plate containing ampicillin and incubated overnight at 37 °C. A colony was picked and a pre-culture was grown over

night at 37°C. Glycerol stocks E. coli BL21(DE3)-ADH-A containing 15% glycerol were prepared from the pre-culture.

2.5.2. Cultivation conditions

A pre-culture with 2x YT media (1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v)NaCl) containing 50 µg/mL ampicillin was inoculated with 1%(v/v) of *E. coli* BL21(DE3)-ADH-A (glycerol stock) and grown over night at 37 °C. Expression was carried out in 2x YT media containing 50 µg/mL ampicillin, inoculated with 1%(v/v) of pre-culture and cells were incubated at 30 °C until an OD600 of 0.4 was reached. Expression of the ADH-A was induced by addition of 1 mM IPTG. The culture was incubated for additional 5 h at 30 °C. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C.

2.5.3. ADH-A purification

The bacteria were re-suspended in 20 mL Binding Buffer (20 mM sodium phosphate, 20 mM imidazole, 500 mM NaCl, 0.02% sodium azide, pH 7.5) fortified with protease inhibitors, lysozyme (2mg/mL) and DNaseI. The cells were lysed using a Cell disruptor and debris was removed by centrifugation at 15000 rpm for 1 h at 4°C. The protein was purified using an Ni^{2+} column, being eluted in the presence of 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, 0.02% sodium azide, pH 7.5. Salts were removed using a PD10 column. The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™). The ADH-A preparation was frozen using liquid nitrogen and stored at -20 °C.

2.5.4. ADH-A activity measurement

The activity of ADH-A was determined by UV assays. The change in absorbance of NADH was monitored at 340 nm under the following conditions:

50 mM Tris buffer (pH 8.0), 5 mM ethyl acetoacetate, 0.2 mM NADH, 0.0011 mg/mL ADH-A. All components were mixed and the reaction was started by addition of the enzyme solution. The change in absorbance was measured at 30 °C and 340 nm. The linear change in absorbance within the first minute of the measurement showed an enzyme activity of 27.53 U/mg for ADH-A.

2.6. *Me*FDH preparation

2.6.1 Bacterial strains and plasmids

The expression construct, pET22b(+)-fdh1B, was transformed into chemically competent *E. coli* BL21(DE3). 50 µL competent cells and approximately 100 ng plasmid used. Transformed cells were grown for 1 h at 37 °C in LB without antibiotics. 100 µL were plated out on a LB plate containing ampicillin and incubated overnight at 37 °C. A colony was picked and a pre-culture was grown overnight at 37°C. Glycerol stocks of *E. coli* BL21(DE3)-fhd1B containing 15% glycerol were prepared from the pre-culture.

2.6.2. Cultivation conditions

A preculture with LB (Lysogeny broth, 100 µg/mL ampicillin) was inoculated with 1%(v/v) of *E. coli* BL21(DE3)-fdh1B (glycerol stock) and grown over night at 37 °C. Expression was carried out in LB media containing 100 µg/mL ampicillin, inoculated with 5%(v/v) pre-culture and cells were incubated at 30 °C

until an OD600 of 0.8 was reached. Expressions of the *Me*FDH was induced by addition of 1 mM IPTG. The culture was incubated for additional 20 h at 25 °C. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C.

2.6.3. MeFDH purification

The bacteria were resuspended in 20 mL Lysis Buffer (50 mM sodium phosphate, 10 mM imidazole, 300 mM NaCl, pH 7.0) fortified with protease inhibitors, lysozyme (2mg/mL) and DNaseI. The cells were lysed using a Cell disruptor and debris was removed by centrifugation at 15000 rpm for 1 h at 4°C. The protein was purified using an Ni^{2+} column, washed with washing buffer (50 mM sodium phosphate pH 7.0, 300 mM NaCl, 20 mM imidazole) and being eluted in the presence of 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 7.0. Salts were removed using a PD10 column anaerobic in the glovebox. The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™). Enzyme preparations were stored at -80 °C anaerobically.

3. SCREENING OF KETOREDUCTASES WITH COFACTOR ANALOGUES, REACTION CONDITIONS

A preliminary screening was performed using recombinantly expressed and lyophilized ADHs from *Lb*ADH from *Lactobacillus brevis*, ADH-A from *Rhodococcus ruber*, *TeS*ADH, *Ras*ADH from *Ralstonia* sp., *Sy*ADH from *Sphingobium yanoikuyae*, ADH-T, *Cp*ADH from *Candida parapsilosis* displaying 0 to 7% conversion by GC analyses. Further screenings were performed as described below.

3.1. Screening of a Codexis ketoreductase (KRED) kit

In a microcentrifuge plastic tube (1.5 mL in volume), the ADH enzyme (10 mg of lyophilized ADH per 1 mL reaction), potassium phosphate buffer (50 mM, pH 7.0; final volume of 1 mL), cofactor BNAH (10 mM, added in acetonitrile, 2% final volume: 20 µL) and cyclohexanone (10 mM) were added. The reaction mixture was placed in a thermomixer at 25 °C and 800 rpm for 24 h. The reaction was stopped through extraction (500 µL of ethyl acetate containing 5 mM dodecane as an internal standard). After centrifugation (13000 rpm, 1 min) and separation of the two phases, the ethyl acetate layer was dried with anhydrous $MgSO_4$, centrifuged (12000 rpm, 1 min), transferred to GC vials and analyzed by GC. Blank reactions were carried out in the absence of BNAH or any type of cofactor (Figure 1 in manuscript).

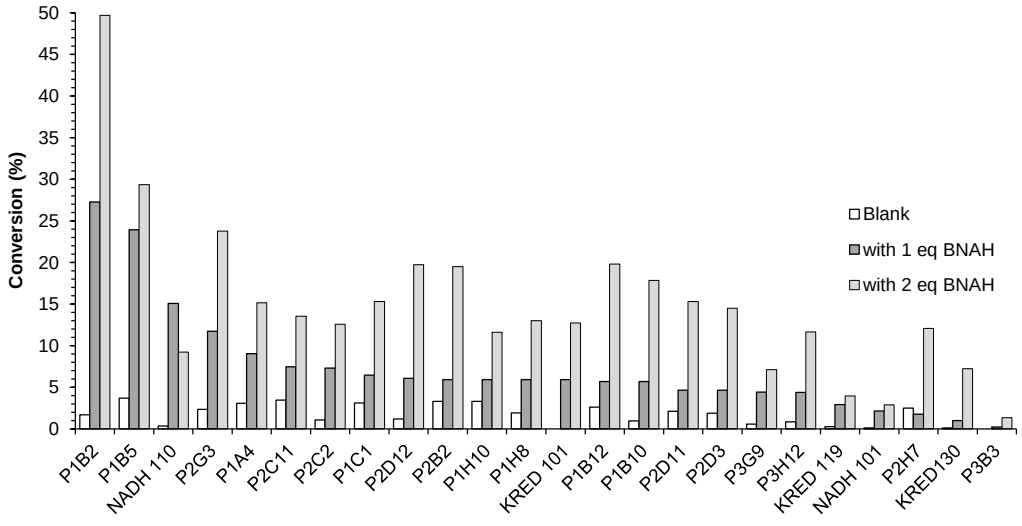


Figure S3. Screening of the ketoreductases (KREDs) Codexis kit. X-axis: KREDs. Y-axis: conversion to cyclohexanol after 24 h, measured by GC analysis, with BNAH (grey) and without BNAH (white). Reactions conditions: 50 mM KPi pH 7.0, [cyclohexanone] = 10 mM, [KRED] = 10 mg/mL, [BNAH] = 10 mM (where applicable), 30 °C. Conditions for 2 equivalents BNAH: 50 mM KPi pH 7.5, [cyclohexanone] = 10 mM, [KRED] = 10 mg/mL, [BNAH] = 20 mM, 25 °C, 18 h.

3.2. Screening with TADH

TADH (ADH from *Thermus* sp. ATN1) was produced and purified as previously described.[8] For the reduction of cyclohexanone to cyclohexanol, the same protocol was used as in section 3.1, with conversions measured by GC analysis. A control reaction with no cofactor added showed no product. Similarly, no product formation was observed with stoichiometric amounts of BNAH. The addition of both BNAH and adenosine monophosphate (AMP) also led to no conversion, whereas catalytic amounts of NAD^+ and stoichiometric amounts of BNAH together led to significant product formation

(12%), leading to the conclusion that BNAH is reducing NAD^+. Furthermore, in the oxidation of cyclohexanol to cyclohexanone, no conversion was observed with stoichiometric amounts of BNA^+.

3.3. Screening of purified ADHs

Enzymes tested:

1) *Hv*ADH2 *Haloferax volcanii* ADH2, $NADP^+$ dependent
2) *Hv*ADH2-F108G variant, $NADP^+$ dependent
3) *Hw*ADH *Haloquadratum walsbyi* ADH, NAD^+/$NADP^+$ dependent
4) HLADH-EE horse liver AD, NAD^+ dependent

Synthetic oxidized cofactors tested at 1, 5 and 10 mM with wild-type *Hv*ADH2 and variant *Hv*ADH2-F108G. No activity was detected under the varying concentrations with 100 mM EtOH (data not shown), in 4 M KCl, pH 10 or pH 8. The inhibitory effect of synthetic cofactors (10 and 20 mM) were tested under standard conditions with 1 mM $NADP^+$, 100 mM EtOH, in 4 M KCl, pH 10 (Figure S4).

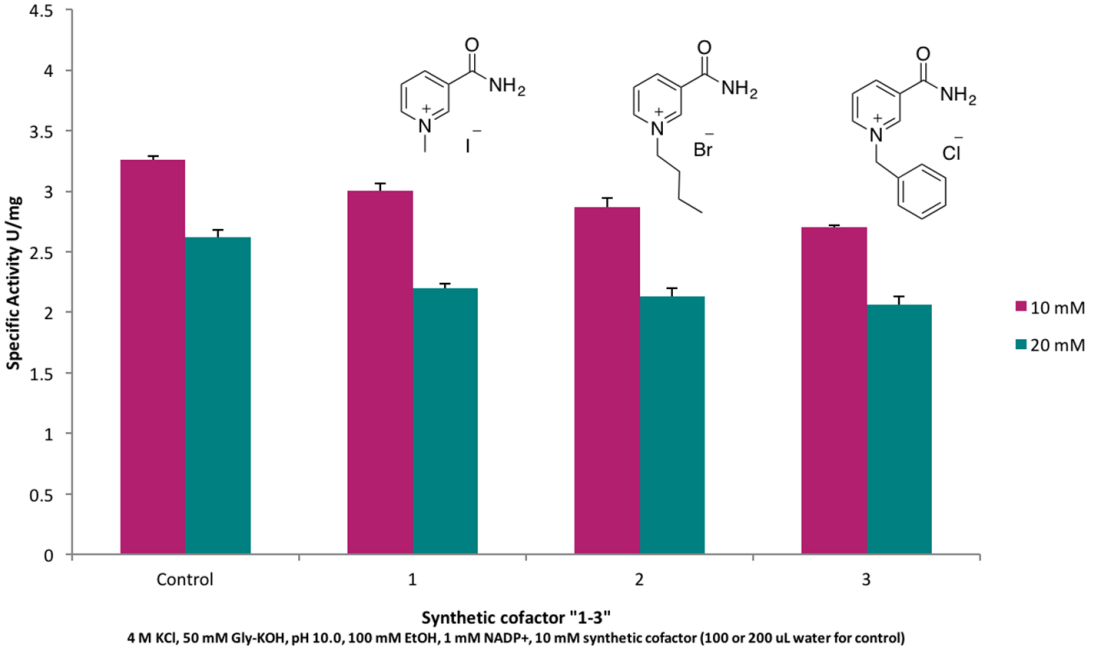
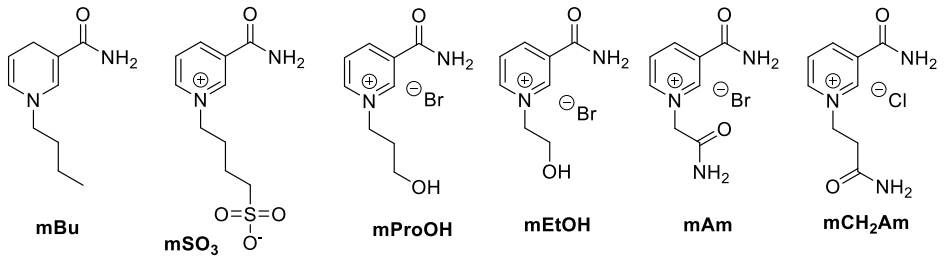


Figure S4. Inhibitory effect of synthetic NCBs on the activity of WT *Hv*ADH2.

Assay conditions:



WT *Hv*ADH2 was tested with 0.1 and 0.5 mM BNAH and mBu in the reduction of 10 mM cyclohexanone in 4 M KCl, pH 8.0 and 10.0. No activity was detected (Table S1). *Hw*ADH (3 M KCl, 50 mM Gly-KOH, pH 10.0) and HLADH were also tested with 0.1 mM BNAH and mBuS in the reduction of 10 mM

cyclohexanone. No activity was detected (Table S1). HLADH (50 mM Tris-HCl, pH 8.8, 4 mM ethanol) were tested with 1 mM mSO_3, mProOH, mEtOH, mAm and mCH_2Am. No activity was detected (Table S1).

Production of the NADPH cofactor was detected at 340 nm, measured in intervals of 1 min for 20 min at 50 ˚C (Epoch 2 microplate reader, BioTek, Bad Friedrichshall, Germany; 96 Well Clear Flat Bottom UV-Transparent Microplate, (Corning®, 3635). All kinetic assays were performed in triplicate. The blank was treated without the addition of enzyme, instead storage buffer.

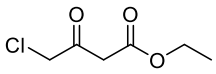
Table S1. Activity of purified ADHs with synthetic NCBs.

ADH enzyme	BNAH red	mBu red	mSO_3 ox	pProOH ox	mEtOH ox	mAm ox	mCH_2Am ox
WT *Hv*ADH2	×	×	×	×	×	×	×
HLADH-EE	×	×	×	×	×	×	×
***Hw*ADH**	×	×	×	×	×	×	×

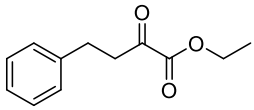
3.4. Screening of a ketoreductase library

A ketoreductase library containing both ADHs and short chain alcohol dehydrogenases/reductases (SDRs) was screened for activity with BNAH. The difference in UV absorbance was measure at 340 nm for BNAH as previously described ($\varepsilon_{340\ nm}$ = 7254 $M^{-1}cm^{-1}$).[9]

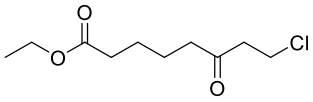
Reaction conditions: potassium phosphate buffer (100 mM, pH 7.0; final volume of 1 mL), enzyme (crude enzyme solution 10 µL), cofactor BNAH (10 mM, added in acetonitrile, 1% final volume) and substrate (200 mM) were added. The reaction mixture was mixed and incubated at 30 °C. The enzyme activity was calculated on the basis of change of the absorbance at 340 nm in 1 min.



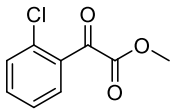
Ethyl 4-chloroacetoacetate (COBE) **1**



Ethyl 2-oxo-4-phenylbutyrate (OPBE) **2**



Ethyl 6-oxo-8-chloroocatanoate (ECOO) **3**



Methyl 2-(2'-chlorophenyl)-2-oxoacetate (CBFM) **4**

Table S2. Screening of a ketoreductase library with BNAH.

Ketoreductase	Substrate	ΔA_{340}/min	Enzyme activity (U/mL)
CgKR1	**1**	-0.0103	-0.17
KaCR1	**1**	0.0079	0.13
PsCR	**1**	0.0014	0.02
Y6	**1**	0.0052	0.08
SCO2	**1**	0.0091	0.15
Y8	**1**	0.0031	0.05
ClCR	**1**	**0.0317**	**0.51**
TBADH	**1**	0.0077	0.12
Y9	**1**	0.0065	0.10
yueD	**1**	**0.0262**	**0.42**
SCO4	**1**	0.0186	0.30

SCO1	**1**	0.0065	0.10
Y10	**1**	0.0124	0.20
ytbE	**1**	**0.0217**	**0.35**
CgCR	**1**	0.0026	0.04
KtCR	**1**	0.0005	0.01
DhCR	**1**	-0.0001	0.00
DhCR-V6	**1**	0.0152	0.24
KIKR	**2**	-0.0072	-0.12
MOSDR	**2**	-0.001	-0.02
CaKR2	**2**	-0.0078	-0.13
CaKR1	**2**	**0.0221**	**0.36**
FabG	**2**	**0.0201**	**0.32**
Cg4	**2**	-0.0021	-0.03
Cg8	**2**	0.0113	0.18
Cg3	**2**	0.0021	0.03
Cg5	**2**	0.0034	0.05
CgKR1-M4	**2**	0.001	0.02
CgKR1-M9	**2**	0.011	0.18
LbCR	**3**	0.0007	0.01
RE2	**3**	0.001	0.02
idols	**4**	-0.0026	-0.04
DaCR	**4**	0.0124	0.20

From the above screening in Table S2, the recombinantly expressed enzymes that displayed highest activity (in green), ClCR, CaKR1, FabG, yueD and ytbE, were selected for further study.

3.4.1. Reactions using wet cells of selected recombinant enzymes

The recombinantly expressed enzymes required different substrates:

Wet cells (100 g/L)	Substrate (10 mM)
yueD	**1** COBE
ClCR	**1** COBE
CaKR1	**2** OPBE
ytbE	**4** CBFM
FabG	**3** OPBE

Reaction conditions: potassium phosphate buffer (100 mM, pH 7.0; final volume of 1 mL), enzyme (100 mg of wet cells 100 g/L), cofactor BNAH (10 mM, 2.1 mg). DMSO (50 µL, 5% volume) and the adequate substrate (10 mM) were added. The reaction mixtures were placed in a thermomixer at 30 °C, 1000 rpm for 12 h. After 12 h, the substrates and products were detected by GC.

Reactions were carried out using whole cells. From the screening of unpurified ketoreductases with BNAH, the recombinantly expressed enzymes ClCR, CaKR1, FabG, yueD and ytbE, were selected for their higher displayed activity (Table S2). Carbonyl reduction reactions were carried out with whole cells using stoichiometric amounts of BNAH with respect to the substrate (10 mM). After 12 h, product was observed with all enzymes, and full conversion was achieved with both FabG and CaKR1. The product formed with FabG had >99% *ee*.
The GC results reveal that ClCR, yueD and ytbE afforded the product (not to completion), whereas FabG and CaKR1 catalyzed the reaction to full conversion. The product with FabG was >99% *ee* (Figure S5), 30% *ee* with CaKR1.

Upon purification of FabG, no product was observed under the same reaction conditions (Figure S6). Therefore within a certain time frame, BNAH could be used in combination with whole cells for the reduction of ketones.

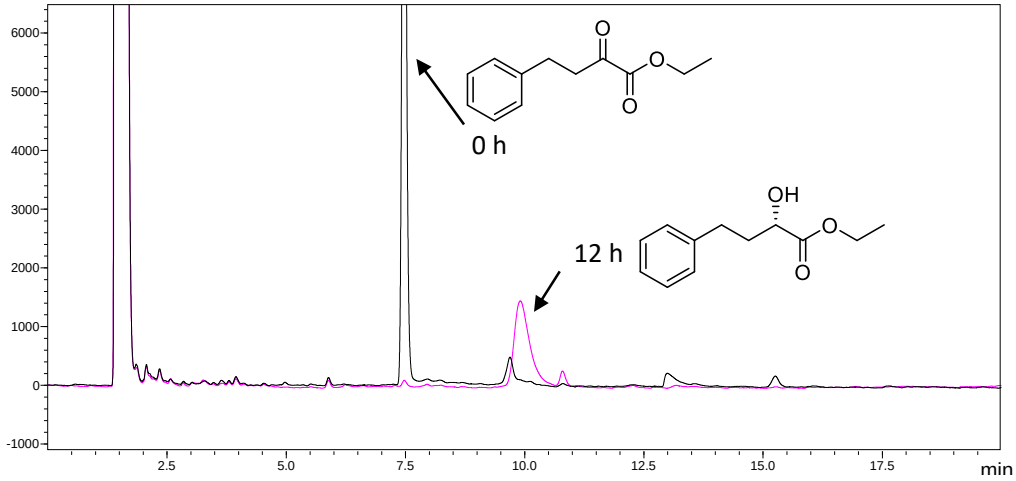


Figure S5. Overlaid gas chromatograms for the enzymatic reaction with FabG after 12 h.

3.4.2. Reaction using the pure enzyme FabG

Potassium phosphate buffer (100 mM, pH 7.0; final volume of 1 mL), pure enzyme of FabG (20 µg/L), cofactor BNAH (10 mM, 2.1 mg). DMSO (50 µL, 5% volume) and substrate OPBE (10 mM) were added. The pH of KPi buffer influenced the results because of the stability of substrate **2** (ethyl 2-oxo-4-phenylbutyrate, OPBE) under different pH. Sample aliquots (100 µl) were taken at different time intervals and mixed with 500 µL of ethyl acetate to extract the substrate and product. The ethyl acetate was dried over anhydrous Na_2SO_4. The enantiomeric excess (*ee*) of the product and the level of conversion were determined by GC analysis.

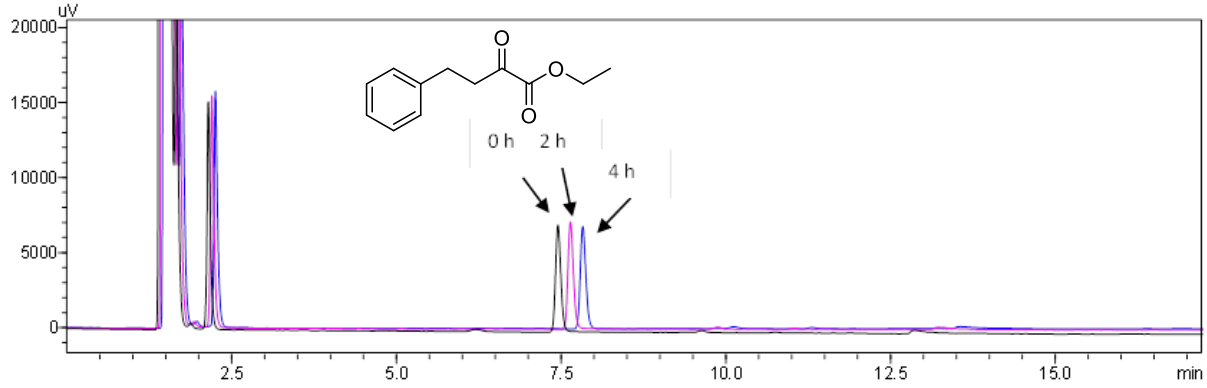


Figure S6. Overlaid gas chromatograms for the enzymatic reaction with FabG at 0, 2 and 4 h. The amount of substrate did not decrease over time, and no product was observed.

4. GC ANALYSES

The progress of the enzyme-catalyzed reactions was followed by GC and all product concentrations were calculated based on calibration curve equations using 5 mM dodecane as an internal standard. GC analyses were carried out on a Shimadzu GC-2010 gas chromatograph equipped with an FID.

For achiral analyses, the following column was used: CP wax 52 CB (50 m × 0.53 mm × 2.0 µm) with the column oven temperature programme: 70 °C for 8 min, 50 °C/min to 120 °C, hold 6 min, 50 °C/min to 240 °C, hold 1 min. Detector temperature: 270 °C, injector temperature: 250 °C. Retention times: cyclohexanone 9.3 min, cyclohexanol 10.9 min.

For chiral analysis of the 4-phenyl-2-butanol product, the following column was used: Chiraldex GTA (50 m × 0.25 mm × 0.12 µm). The injection temperature was 250 °C, helium was used as a carrier gas; linear velocity: 36 cm/s, column flow: 2.01 mL/min, split ratio: 100. Column oven temperature programme: 90 °C for 10 min, 5 °C/min to 110 °C, hold 10 min, 5 °C/min to 120 °C, hold 6 min, 10 °C/min to 170 °C, hold 2 min. The calibration curves using 5 mM dodecane as an internal standard were linear in the range of product detection (R^2>0.999). The HLADH enzyme is known to be (*S*)-selective, therefore the major product was assumed to be the (*S*)-enantiomer. Retention times: dodecane 13.0 min, **4-phenyl-2-butanone** 29.2 min, (*S*)-4-phenyl-2-butanol 31.1 min, (*R*)-4-phenyl-2-butanol 31.6 min. For **phenylacetone**: 80 °C for 10 min, 5 °C/min to 90 °C, hold 10 min, 5 °C/min to 110 °C, hold 7 min, 10 °C/min to 170 °C, hold 2 min. Retention times: dodecane 18.7 min, phenylpropanol (*S*)-32.3 min, (*R*)-32.7 min, phenylacetone 33.4 min. For **2-pentanone**: 60 °C for 10 min, 10 °C/min to 170 °C, hold 2 min. Retention times: (*S*)-2-pentanol 7.4 min, (*R*)-2-pentanol 7.7 min, 2-pentanone 10.8 min, dodecane 18.5 min.

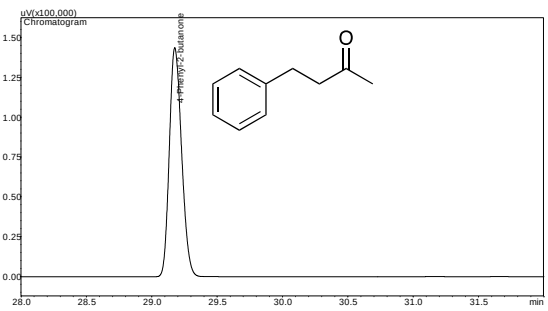
For the analyses of the ketone substrates **1-4** to their corresponding alcohol products the chiral column CP-Chirasil Dex CB with an injection and detector temperature of 280 °C was used. For substrate **1** (COBE): column oven temperature at 140 °C, for substrate **2** (OPBE), at 160 °C, for substrate **4** (CBFM) at 180 °C.

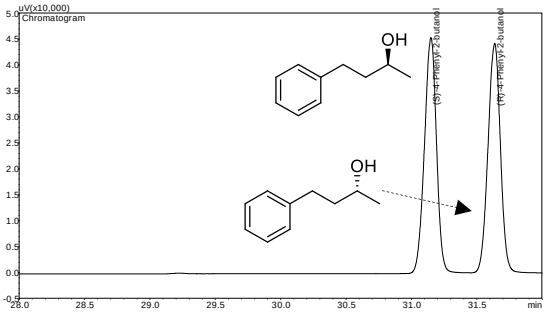
For the analyses of ethyl acetoacetate and the corresponding ketoreduction product ethyl-3-hydroxybutyrate the CP-wax 52 CB GC column (25 m x 0.25 mm x 1.2 µm) was used with the column oven temperature programme: 90 °C for 5 min, 5 °C/min to 101 °C, hold 1 min, 5 °C/min to 135 °C, hold 3 min, 10 °C/min to 145 °C, hold 1 min, 30 °C/min to 250 °C, hold 1 min. Detector temperature: 275 °C, injector temperature: 250 °C. Retention times: dodecane 7.8 min, ethyl acetoacetate 16.8 min, ethyl-3-hydroxybutyrate 19.0 min

.

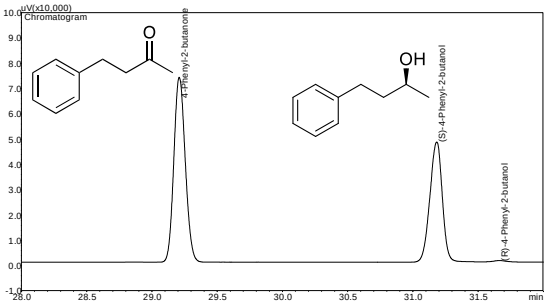
GC chromatograms

Starting material 4-phenyl-2-butanone (left), racemic alcohol product 4-phenyl-2-butanol (right):

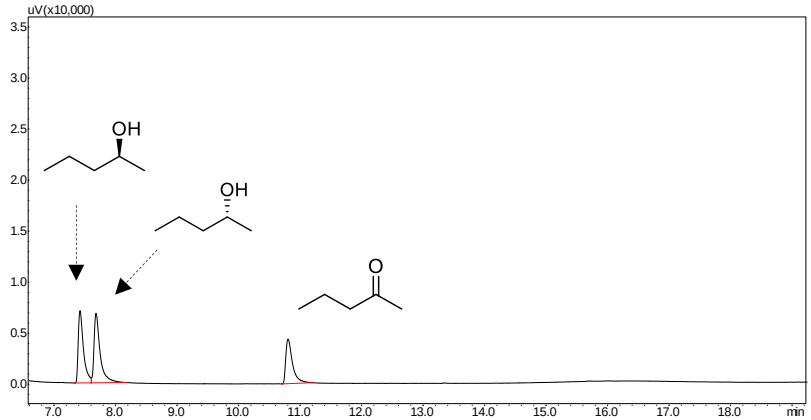


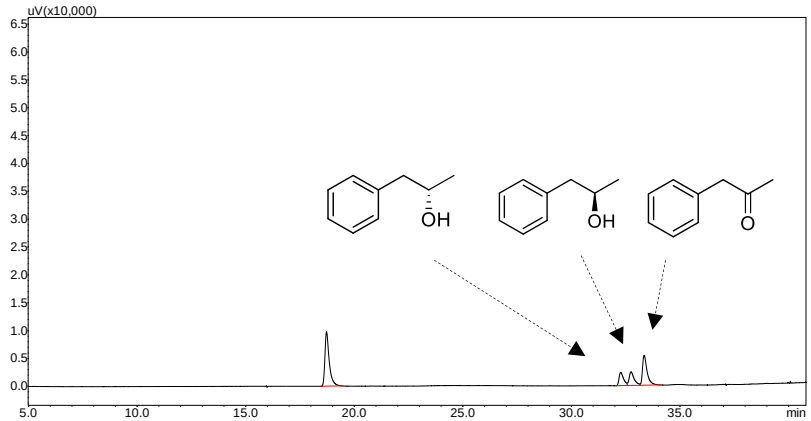


HLADH-catalyzed reaction mixture:



Mixture of substrates and products:





5. RECYCLING SYSTEM

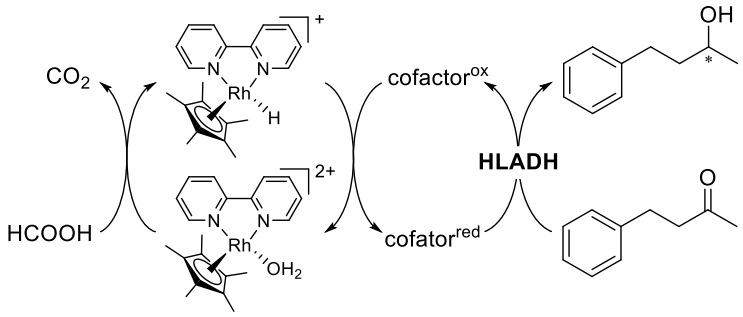
5.1. Standard reaction procedure for the NADH recycling system with the Rh complex

The activity of the Rh complex was measured by UV spectroscopy:

- 10.4 TOF (h^{-1}) at 30 µM $[Cp^*Rh(bpy)(H_2O)]^{2+}$, 50 mM sodium formate, 0.1 mM NAD^+ in 50 mM KPi pH 7.0;
- 19.6 TOF (h^{-1}) at 0.1 mM $[Cp^*Rh(bpy)(H_2O)]^{2+}$, 100 mM sodium formate, 1 mM NAD^+ in 50 mM KPi pH 7.0.

For the biocatalytic recycling experiments, stock solutions of NAD^+, NMN^+ and BNA^+ (10 mM), $[Cp^*Rh(bpy)(H_2O)]Cl_2$, $[Cp^*Rh(bpy)(H_2O)]OTf_2$ (5 mM), sodium formate (1 M) and HLADH (3 mg, 2 U/mg) in deoxygenated phosphate buffer (100 mM, pH 7.0) were freshly prepared before the experiments.

In a microcentrifuge plastic tube (2 mL in volume), potassium phosphate buffer (100 mM, pH 7.0; final volume of 1 mL), cofactor (2.2 mM or none), $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (104 µL, 0.52 mM), sodium formate (52 µL, 52 mM), HLADH (200 µL, 2 U), and substrate 4-phenyl-2-butanone (2.5 µL, 16.7 mM) were added under nitrogen flow. The reaction mixture was placed in an Eppendorf thermomixer at 30 °C and 600 rpm for 18 h, or other indicated times. The reaction was stopped at the indicated time intervals through extraction (500 µL of ethyl acetate containing 5 mM dodecane as an internal standard). After centrifugation (13000 rpm, 1 min) and separation of the two phases, the ethyl acetate layer was dried with anhydrous $MgSO_4$, centrifuged (12000 rpm, 1 min), transferred to GC vials and analyzed by chiral GC.



Scheme S2. Rh complex recycling system.

Table S3. Various reaction conditions for the reduction of 4-phenyl-2-butanone.

	Rh (mM)	$NaCO_2H$ (mM)	HLADH crude	HLADH dialyzed	cofactor (mM)	conv (%)	*ee* (%)
1	0.52	52	2 U		NAD^+	38	98
2	0.52	52		2 U	NAD^+	42	90
3	0.52	52	2 U		NMN^+	4	22
4	0.52	52		2 U	NMN^+	23	<1
5	0.52	52	2 U		BNA^+	4	37
6	0.52	52		2 U	BNA^+	18	<1
7	0.52	52	2 U			11	4
8	0.52	52		2 U		78	<1
9	0.52	52				89	<1
10	0.52	52			NAD^+	37	<1
11	0.52					<1	n.d.
12		52				<1	n.d.

13	0.52	52	4 U		NAD^+	6	91
14	0.52	52	10 U		NAD^+	<1	72
15[a]	0.52	52	2 U		NAD^+	<1	n.d.
16			2 U			<1	94
17			2 U		BNA^+	1	<99
18				2 U	BNA^+	<1	n.d.
19[b]				2 U	NAD^+	14	97
20[c]				2 U		5	>99
21			2 U		NADH + BNA^+	31	96
22			2 U		NADH + BNAH	38	97

[a] no substrate; [b] with 5% v/v ethanol and 0.1 mM NAD^+; [c] with 5% v/v ethanol; n.d. = not detected; conv = conversion; Rh = $[Cp^*Rh(bpy)(H_2O)]^{2+}$. Reaction conditions: 100 mM KPi pH 7.0, 1 mL final volume, [cofactor] = 2.2 mM, $[Cp^*Rh(bpy)(H_2O)]^2$, [sodium formate], HLADH, [4-phenyl-2-butanone] = 16.7 mM, shaken at 30 °C, 800 rpm for 18 h.

Results and discussion of Table S3:

- Entries 1-8 show the Rh catalyzed recycling with cofactors NAD^+, NMN^+ and BNA^+ and in the absence of cofactor (show in manuscript Figure 4). The conversion observed without cofactor and <5 *ee* with crude and dialyzed enzyme shows the Rh complex can directly reduce the substrate.

- Entries 9-12 show no cofactor recycling in the absence of sodium formate (entry 11) or in the absence of the Rh catalyst (entry 12).

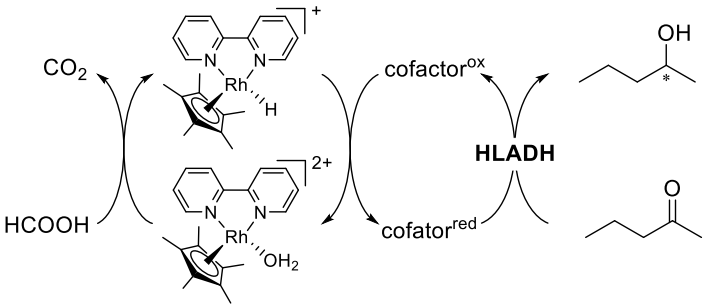
- Entries 13-14 with increased enzyme loading there is a decrease in conversion and *ee*, due to mutual inactivation of the enzyme and Rh complex.

- Entry 15 control reaction without substrate no product is observed.

- Entry 16-18 enzyme and substrate alone show close to 1% conversion and 94% *ee*, then with BNA^+ added 1% conversion, the same reaction with dialyzed enzyme no product is observed.

- Entries 19 and 20 show the NAD^+ is recycled with the addition of 5% v/v EtOH: either with 0.1 mM NAD^+ added (entry 19), or without additional NAD^+ (entry 20), thus after dialysis we cannot completely exclude the presence of NAD^+.

- Entries 21 and 22 indicate no cofactor inhibition from BNA^+ or BNAH.



Scheme S3. Reaction with 2-pentanone as substrate.

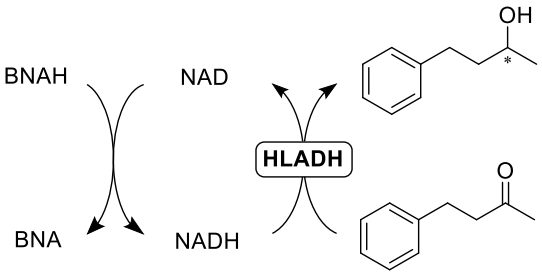
Control reactions performed:

1) Reaction without substrate: no peaks observed on the GC chromatogram.

2) Reaction without cofactor: 13% racemic product (<5% *ee*) observed.

5.2. NADH recycling system with BNAH

The recycling of NAD^+ by BNAH was investigated with the dialyzed HLADH.



Scheme S4. Recycling of NADH with BNAH.

Additional reactions:

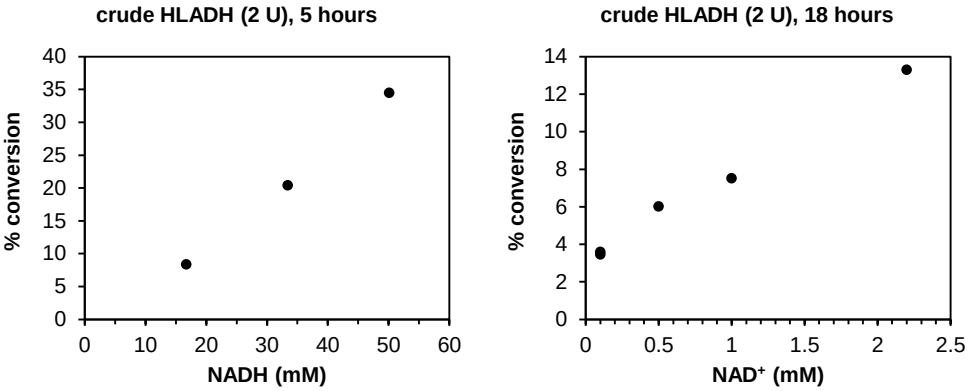


Figure S7. Left: increasing concentration of NADH. Right: increasing concentration of NAD^+.

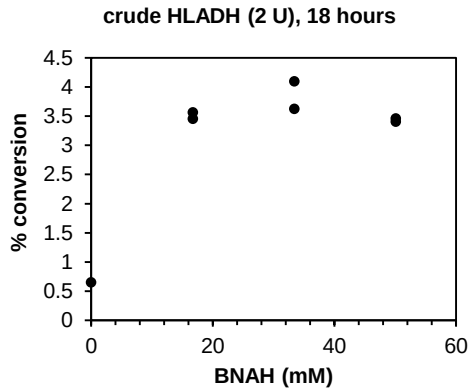


Figure S8. Recycling NADH with BNAH after 18 hours: increasing BNAH concentrations, 0.1 mM NAD^+.

5.2.1 NADH recycling system with BNAH and MeFDH

In a microcentrifuge plastic tube (2 mL in volume), potassium phosphate buffer (50 mM, pH 7.0; final volume of 2 mL), ADH-A (0.6 U), [MeFDH] = 5 µM or 0 µM, [NAD^+] = 0.1 mM, [BNAH] = 10 mM (solid), [ethyl acetoacetate] = 10 mM were added under anaerobic conditions in the glovebox. The reaction mixture was placed in an Eppendorf thermomixer at 30 °C and 800 rpm for 24 h anaerobic in the glovebox. The reaction was stopped at the indicated time intervals through extraction (200 µL of ethyl

acetate containing 5 mM dodecane as an internal standard). After centrifugation (13000 rpm, 1 min) and separation of the two phases, the ethyl acetate layer was dried with anhydrous $MgSO_4$, centrifuged (12000 rpm, 1 min), transferred to GC vials and analyzed by GC.

5.2.2. Reduction and oxidation reactions with HLADH

HLADH-catalyzed reactions were investigation in both direction, reduction and oxidation.

Reduction reactions were run with three substrates: 4-phenyl-2-butanone, phenylacetone and 2-pentanone. Reaction conditions: 16.7 mM of substrate, adding one equivalent of reduced cofactors 1) NADH, 2) BNAH, or 3) BNAH with 1 mM NAD^+, crude HLADH (2 U), 5 h, 30 °C (Table S4).

Table S4

Substrate:	**4-Phenyl-2-butanone**		**Phenylacetone**		**2-Pentanone**	
Cofactor	**Conv. (%)**	***ee* (%)**	**Conv. (%)**	***ee* (%)**	**Conv. (%)**	***ee* (%)**
NADH	23	98	6	>99	21	92
BNAH	<1	n.a.	<1	n.a.	<1	n.a.
BNAH + 1 mM NAD^+	6	98	<1	n.a.	1	n.a.

n.a.= not applicable

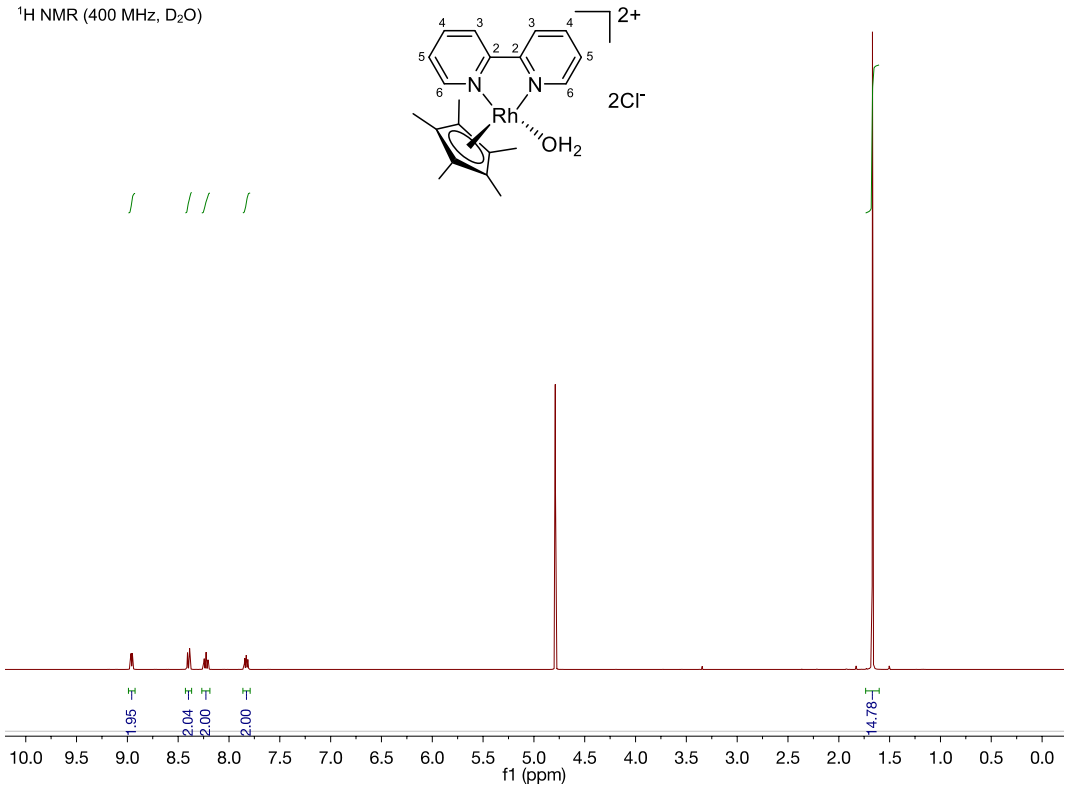
Oxidation reactions were run with 4-phenyl-2-butanol. Reaction conditions: 16.7 mM of 4-phenyl-2-butanol, adding oxidized cofactors, crude HLADH (2 U), 5 h, 30 °C (Table S5).

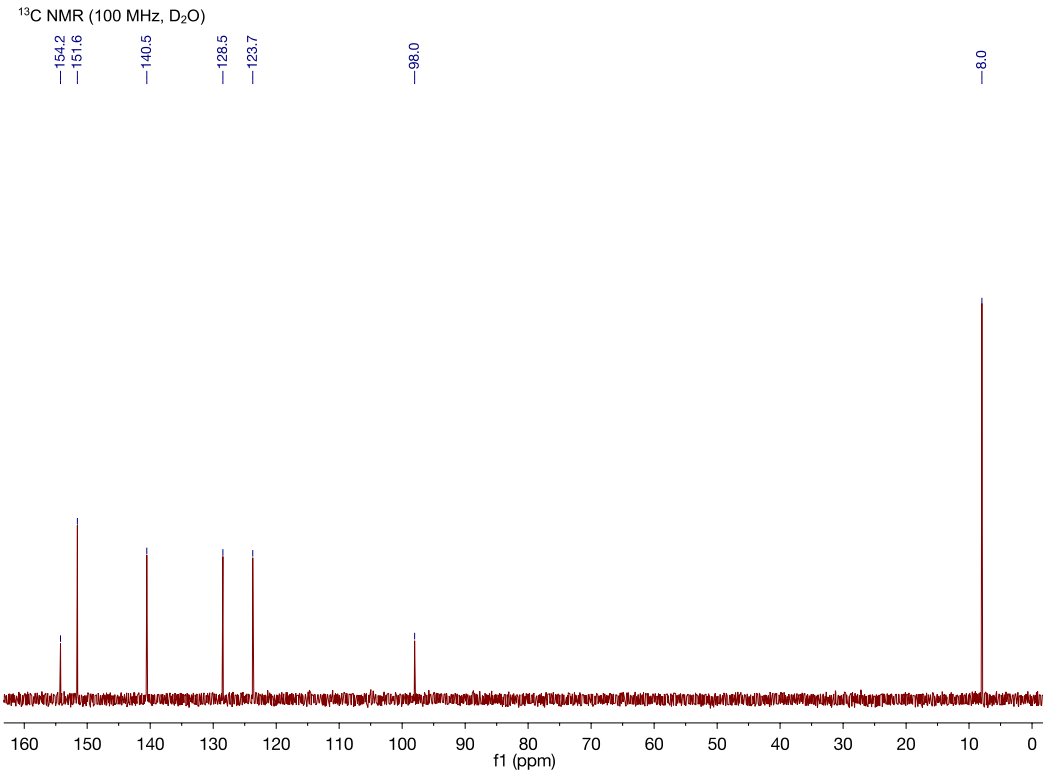
Table S5

	4-Phenyl-2-butanol	
Cofactor	**Conv. (%)**	***ee* (%)**
0.1 mM NAD^+	11	98
16.7 mM NAD^+	22	98
16.7 mM BNA^+	1	n.a.
no cofactor	1	n.a.

6. NMR SPECTRA

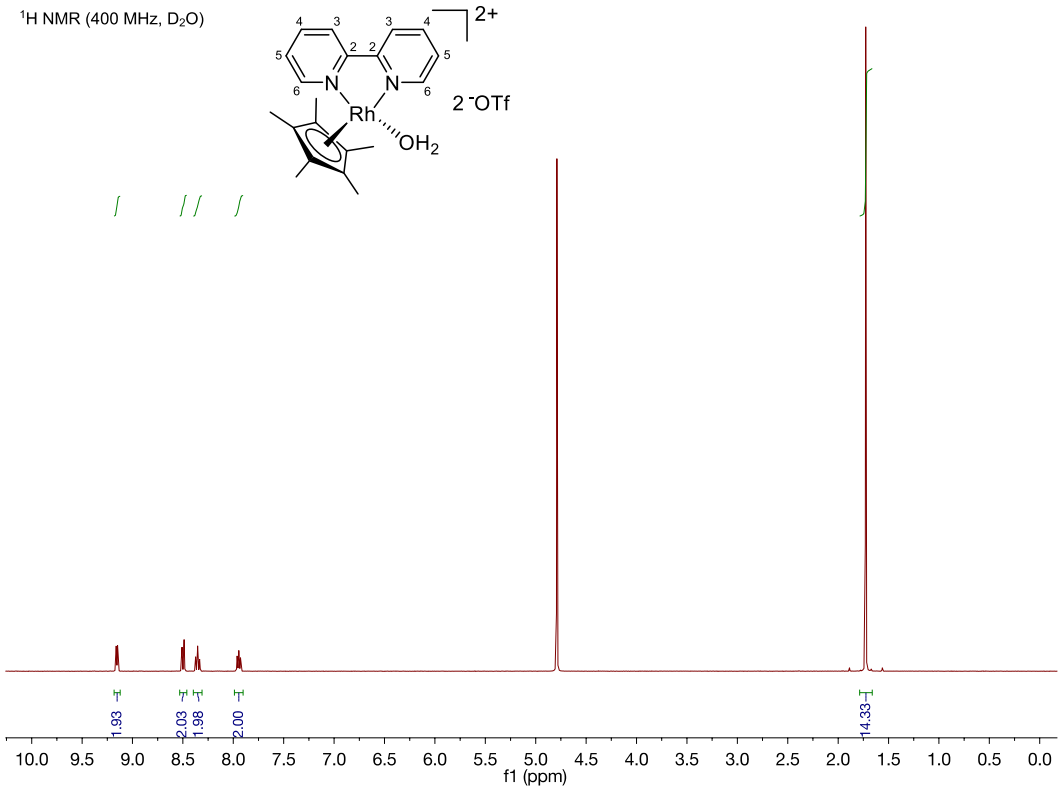
Pentamethyl cyclopentadienyl (2,2'-bipyridyl) rhodium(III) chloride [Cp*Rh(bpy)(H_2O)]Cl_2



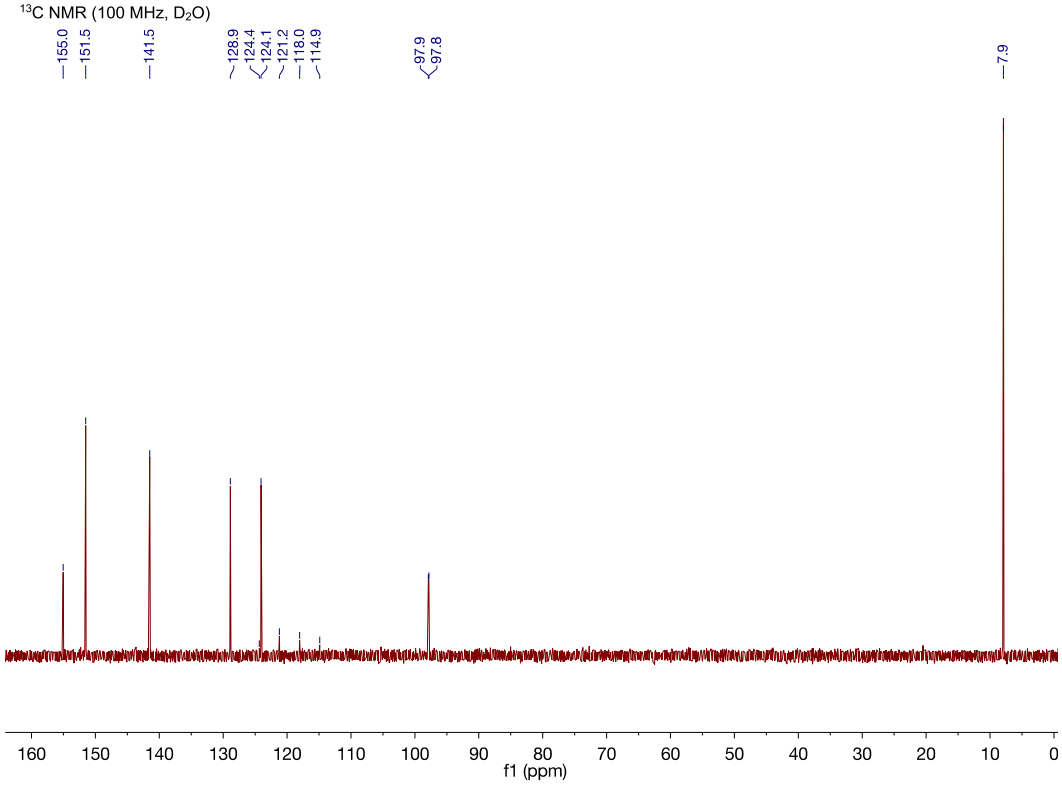


Pentamethyl cyclopentadienyl (2,2'-bipyridyl) rhodium(III) triflate [Cp*Rh(bpy)(H_2O)]($OTf)_2$

^{1}H NMR (400 MHz, D_2O)

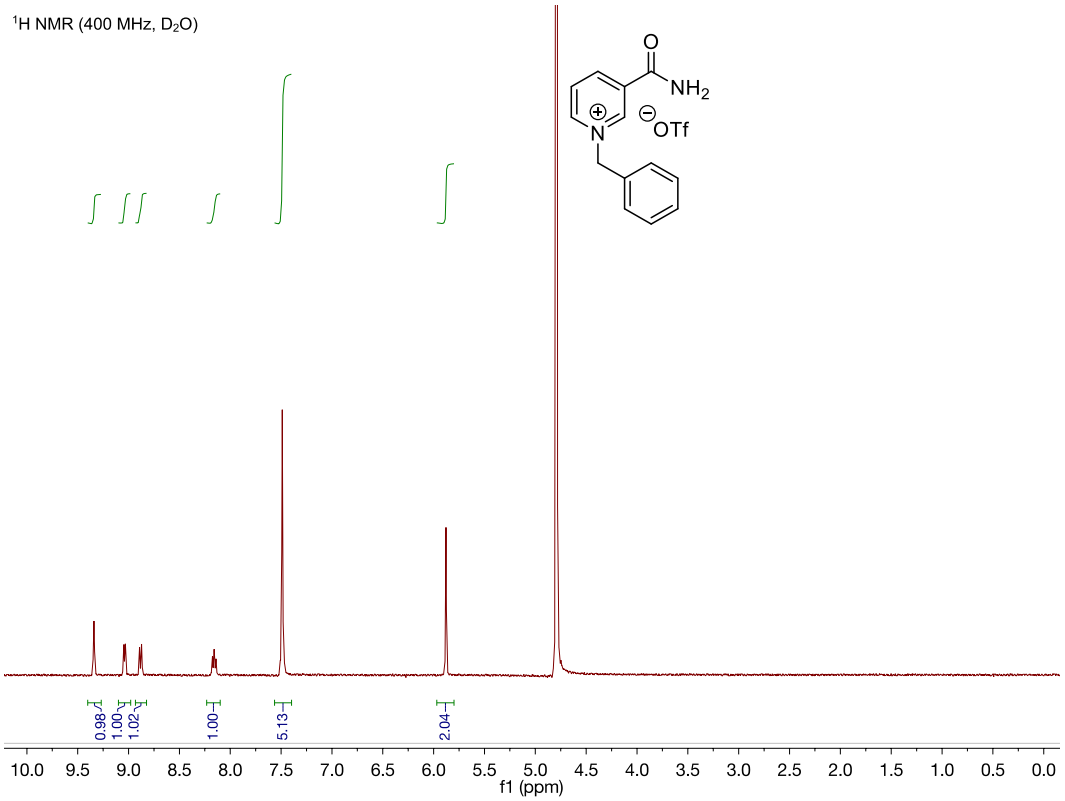


^{13}C NMR (100 MHz, D_2O)

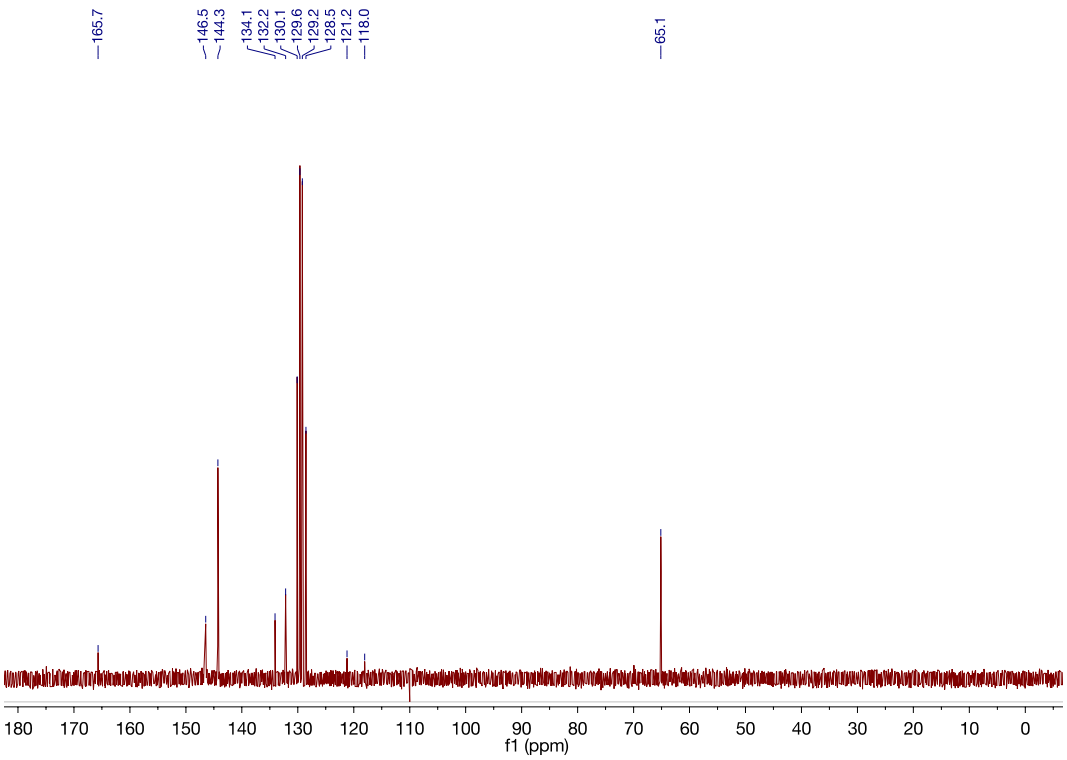


1-Benzyl-3-carbamoylpyridinium triflate BNA^{+} $^{-}$OTf

^{1}H NMR (400 MHz, D_2O)

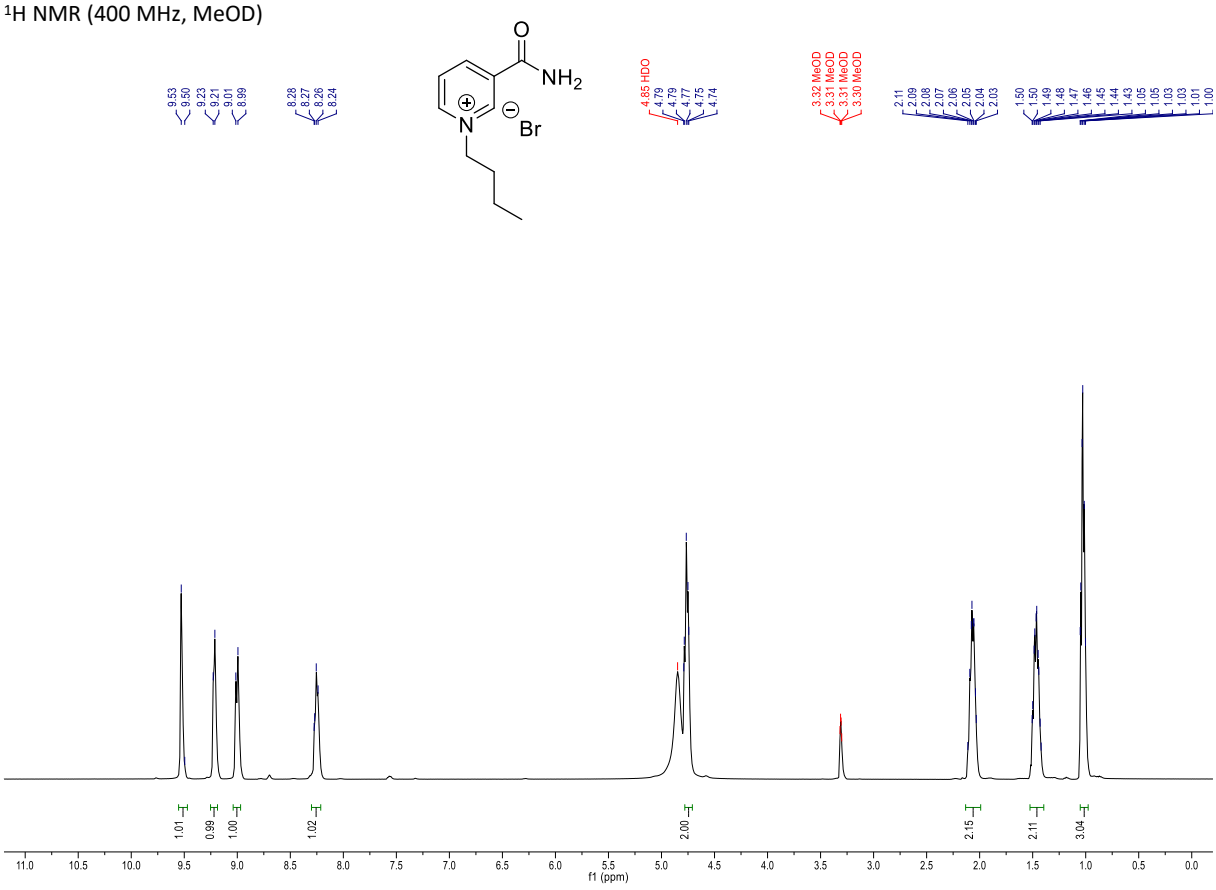


^{13}C NMR (100 MHz, D_2O)



1-butyl-3-carbamoylpyridinium bromide mBu

^{1}H NMR (400 MHz, MeOD)



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