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FULL PAPER

Synthesis and Biochemical Evaluation of Nicotinamide Derivatives as NADH analogues in Ene Reductase

Natashya Falcone, [a, b] Zhe She, [b] Jebreil Syed, [b] Alan Lough, [c] and Heinz-Bernhard Kraatz*[a, b, c]

Abstract: Nicotinamide and pyridine-containing conjugates have attracted a lot of attention in research as they have found use in a wide range of applications including as redox flow batteries, calcium channel blockers, and in biocatalysis, and metabolism. The interesting redox character of the compounds' pyridine/dihydropyridine system allows them to possess very similar, if not able to mimic, the functions and characteristics of the natural chiral redox agents NAD^+/NADH. Considerable interest has been given in designing and synthesizing NAD^+/NADH mimics with similar redox properties. In this research, three nicotinamide conjugates were designed, synthesized and characterized. Molecular structures obtained through X-ray crystallography were obtained for two conjugates providing more detail into the bonding and structure of the compounds. The compounds were then further evaluated for biochemical properties and it was found that one of the conjugates possessed similar functions and characteristics to the natural NADH compound. Compound **4** was evaluated in the active enzyme, Enoate Reductase and compared to NADH, it was shown to be successful in reducing the C=C double bond of three substrates and outperformed the natural coenzyme, kinetic data has been reported.

Introduction

Nicotinamide and pyridine-containing conjugates have attracted a lot of attention in the past years due to their variety of applications in different fields, from playing a key role in metabolism, to being an electron carrier in many biological pathways, and for their roles in medicinal scaffolds and as redox flow batteries.[1–6] The most common and biologically significant nicotinamide conjugate is NAD^+/NADH, nicotinamide adenine dinucleotide, which is a cofactor involved in over 300 biochemical reactions and biological processes including redox reactions, cellular metabolism, and cellular respiration.[7,8] Nicotinamide conjugates containing the pyridine/dihyropyridine system, which NAD^+ possesses, are the closest analogues to the natural cofactor found in nature. It has been reported that dihydropyridine compounds are unique in the critical sense that it is a catalytic hydride donor similar to NADH rather than a stoichiometric hydride reagent.[9] This is due to the fact that the pyridines catalytic behaviour lies in the chemistry of the 1,2-dihydropyridine/pyridine redox couple driven by a dearomatization-aromatization process. The biologically important redox couple provides a reversible formation of a C-H bond centered on the nicotinamide ring representing an efficient system for numerous biological hydrogen-transfer reactions.[9] Considerable interest has been given in creating these nicotinamide conjugates that are able to mimic the activity of the NAD^+/NADH redox couple[10,11] and mimic the activity from the viewpoints of the electrochemical behavior. Many groups have studied the electrochemical behaviour and regeneration of NAD^+/NADH.[12–15] Sanford et al. designed and synthesized various pyridine containing compounds and used its redox properties in the application of redox flow batteries.[16] They reported the evolutionary design of a series of pyridine-based anolyte materials that exhibit up to two reversible redox couples at low potentials in the presence of Li-ion supporting electrolytes. The Colbran group reported the synthesis of pyridine/dihydropyridine conjugates for bio-inspired reduction and multi-electron reduction,[17] and demonstrated that a combination of an organic hydride pyridinium compound and a metal center can complex and lead to an efficient catalyst for transfer hydrogenation. In addition, Musgrave et al. reported the reduction of CO_2 to methanol catalyzed by a biomimetic organo-hydride produced from pyridine, where the 1,2-dihydropyridine acts as a recyclable organo-hydride that reduced CO_2 to methanol via three hydride and proton transfer steps.[18] Dihydropyridine compounds have also been heavily reported to act as calcium channel blockers and are explored for the development of pain therapeutics.[4,20] Given the potential application in biocatalysis, it is not surprising that the synthesis of these pyridine conjugates have attracted the attention of numerous researchers for years.[21–27]

Not only has considerable interest been given in creating model compounds that mimic the activity of the NAD^+/NADH redox couple, but mimics containing the nicotinamide moiety have been synthesized and tested in active enzymes as replacements to the natural cofactors. Lowe et al. developed an artificial coenzyme, CL4, which retained the nicotinamide portion and replaced the phosphate and adenine moieties with other functional groups. They showed its activity in the active enzyme horse liver alcohol dehydrogenase (HLADH).[28] A couple years later Fish et al. demonstrated that a simpler nicotinamide coenzyme mimic, also retaining the nicotinamide portion and substituting the rest with a simple benzyl group, displayed activity in HLADH.[29,30] This group continued to report on these mimics being able to catalyze the reduction of aldehyde compounds to chiral alcohol products and for other hydrogenation applications.[30–33] Through many studies it was demonstrated that the nicotinamide moiety of NAD^+ is all that is necessary for enzyme recognition and redox function.[29,34,35] This concept was

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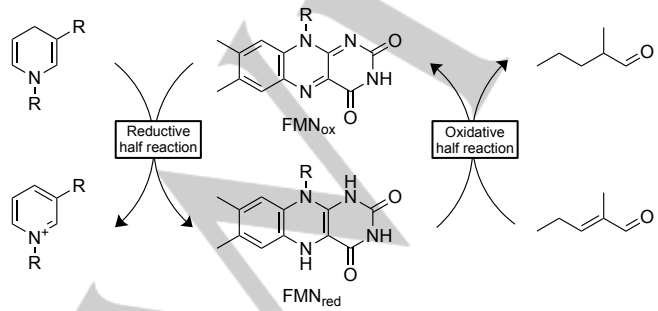
later further applied by Clark et al. in cytochrome P450 enzymes where they engineered heme-containing enzymes for improved activity towards the same biomimetic cofactor.[36] In addition, Paul and Hollman designed and evaluated a range of NADH biomimics in enoate reductase (ER) enzymes.[34,37] Although biomimics of NAD(H) have been developed and have found applications within chemo-enzymatic synthesis, to date, only a few examples have been published including reports on kinetic data for reductases.[34,37,38] In addition, many of the reported NADH mimics mentioned above are similar in structure and other functional groups conjugated to the pyridine ring have not been explored for further alterations.

Herein, we report the synthesis and characterization of three nicotinamide-containing conjugates. These were characterized, including two of the compounds, which were characterized by single X-ray crystallography, allowing the determination of intra- and intermolecular interactions. Specifically, these NAD-mimics were designed with future modifications in mind that may allow further alterations and conjugations. From the X-ray structures we observe the engagement of intermolecular bonding between neighboring nicotinamide moieties through the carbonyl and amide nitrogen functional groups with hydrogen bonding distances in a comparable range to the natural cofactor. Through electrochemical evaluation of the compound we found that the three conjugates also all exhibit a characteristic NADH reduction peak of compounds at -1.1-(-1.6) V. [12,13] We then further explored the reduction of the compounds into their corresponding dihydropyridine derivatives. This was followed by enzymatic transformations using the dihydropyridine derivatives as hydride transfer agents involving the enzyme ER, a yeast recombinant made in *E. coli* (EC 1.3.1.16), as represented in Scheme 1. ERs are a class of redox enzymes that are capable of catalyzing the asymmetric reduction of activated C=C bonds forming stereogenic centers.[39–41] The ability to catalyze reductions and the wide acceptance of different substrates are driving the exploitation of ERs towards novel applications in redox biocatalysis and their implementation in industrial processes.[42a] The ER catalytic reaction resembles an asymmetric Michael-type addition of a chiral hydride to an enone. The catalytic cycle can be divided into two separated half reactions. In the first, a hydride is transferred from NADH to the enzyme-bound flavin.[34] After the release of the oxidized NAD^+, the hydride is then transferred from the bound flavin to the activated alkene substrate (Scheme 1).



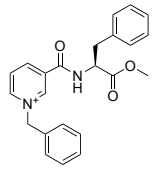
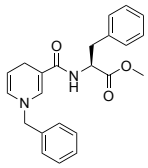
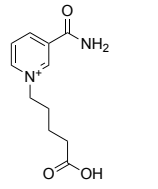
Scheme 1. Scheme showing the catalytic cycle of ER using nicotinamide compounds as the coenzyme and 2-methyl-2-pentenal as the substrate. In the reductive half reaction, a hydride is transferred from NADH (or a mimic of). NADH oxidation can be followed spectroscopically by monitoring decrease of the characteristic absorption band at 340-360 nm. In the oxidative half reaction, the hydride is then transferred from the bound-flavin, oxidizing the flavin, to the alkene substrate for C=C reduction.

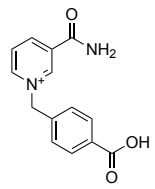
Compound **4** displayed biological activity with ER allowing us to drive the enzymatic transformation of three enoate substrates. Here, we report a class 2 Ene reductase (see SI) that accepts a nicotinamide coenzyme biomimetic with a side by side comparison of kinetics to the natural coenzyme, NADH. We provide details of this study including the design and synthesis of the compounds, the biochemical evaluation of them, and detailed enzyme kinetic analysis.

Results and Discussion

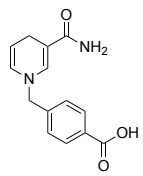
Three pyridine-containing conjugates were synthesized using nicotinamide and nicotinic acid as the starting materials. Conjugate **1**, a phenylalanine derivative, was synthesized by peptide coupling, followed by pyridine-modification using benzyl bromide. Conjugates **2** and **3**, used nicotinamide as the starting material and were synthesized by a simple one-step reflux reaction with 5-bromo valeric acid and 4-bromomethyl benzoic acid, respectively. It was reported that the nicotinamide moiety is all that is necessary for enzyme recognition and for the involvement in the oxidation/reduction process, while the adenine moiety infers catabolic from anabolic reactions which is irrelevant in catalytic applications. All three compounds retained the nicotinamide moiety and modifications at the pyridine nitrogen and amide nitrogen were explored. These compounds were chosen with further modifications in mind including modifications for surface studies, specifically, for compounds **2** and **3** by adding the additional carboxylic acid functional group to the reported benzyl group**.** The three nicotinamide-containing compounds and corresponding dihydropyridines are reported in Table 1 and characterization can be found in the SI.

Table 1. Table showing the molecular structure of compounds **1** - **5** and their corresponding dihydropyridine.

Compound	Corresponding dihydropyridine
 **(1)**	 **(5)**
 **(2)**	N/A



(3)



(4)

corresponding product **4** and **5**, while no discernable reduction products involving **2** were obtained. (See Table 1) This is surprising since all three conjugates are reducible electrochemically (*vide infra*). At this point, we can only speculate that the aliphatic substituent on the pyridine nitrogen interferes with the chemical reduction using dithionite. Potential differences in the electronic interactions between the benzoic acid versus valeric acid may play a role, since reductions using dithionite can be dependent on electron withdrawing groups.[42b,c,d] Both

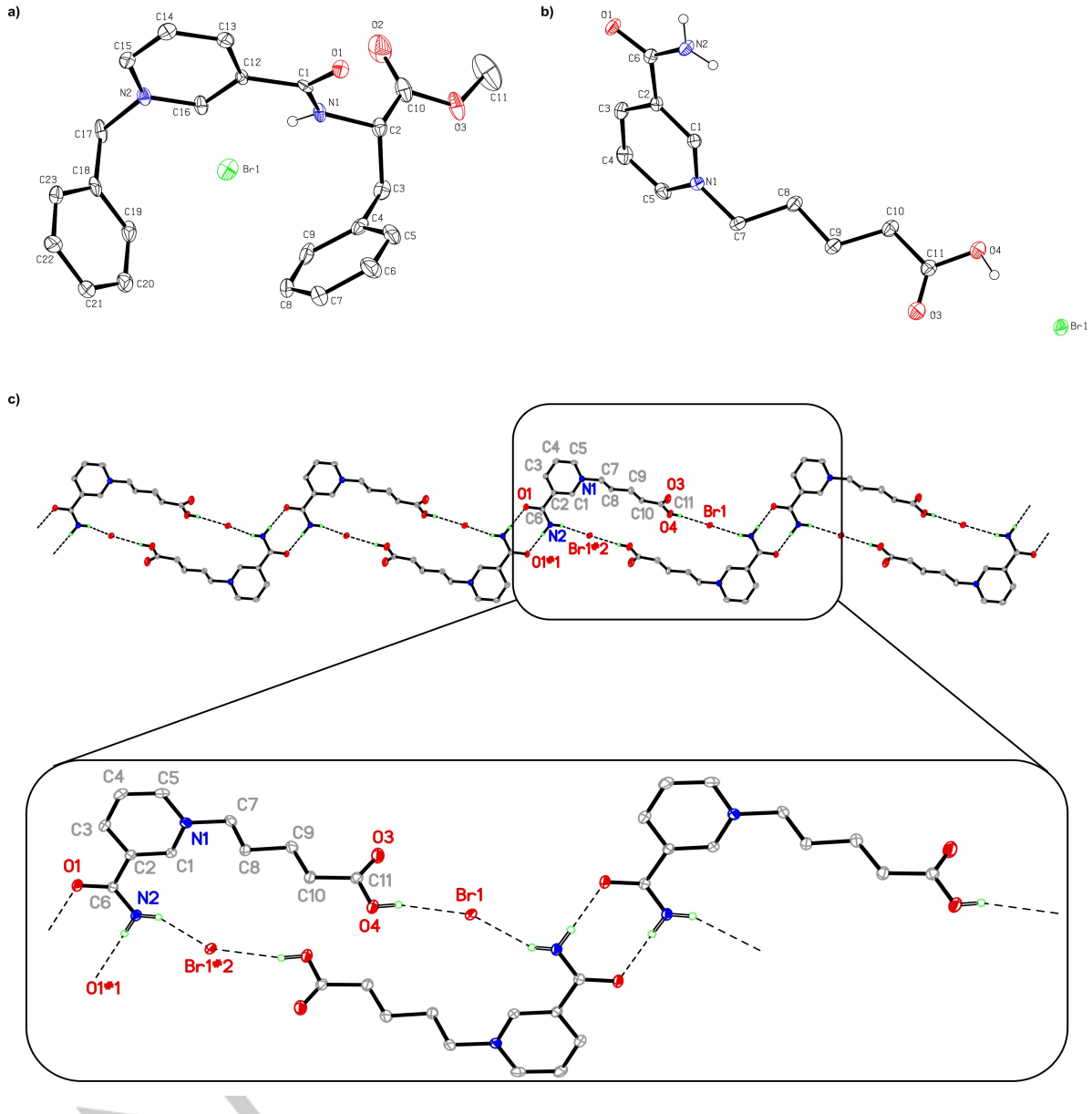


Figure 1. Molecular structure obtained by single crystal X-ray crystallography for A) Compound **1** and B) Compound **2.** c) Hydrogen bonding interactions in compound **2** involving the O4, O1, N2, and Br1 atoms between adjacent molecules, d[N2—O1] = 2.870(3) Å, d[N2-Br1] = 3.356(2) Å, and d[O4—Br1] = 3.1811(19) Å, resulting in the formation of a one-dimensional chain exhibiting a zig-zag pattern.

Reduction of compounds **1-3** was explored using sodium dithionite as the reducing agent to form the corresponding 1,2-dihydropyridine. We are able to reduce conjugates **1** and **3** to the compounds show a strong absorption λ_{max} = 360 nm which is characteristic of dihydropyridines, and which is also present for

NADH at 340 nm.[43] The UV spectra of compounds **1, 3, 4** and **5,** can be seen in Figure **S22.**

Electrochemical reduction of the nicotinamide conjugates **1** - **3** was also explored by cyclic voltammetry (CV).[12,13] All CV experiments were carried out in a 1 mM solution of the compounds in 100 mM phosphate buffer (pH 7.05). A glassy carbon working and counter electrode was used with reference to a Ag/AgCl reference electrode. At a scan rate of 100 mV/s, the cyclic voltammograms of the compounds exhibited a reduction signal at -1.1 - -1.6V shown in Figure **S21,** which is characteristic of reduction of the nicotinamide moiety.[12,13,44,45a]

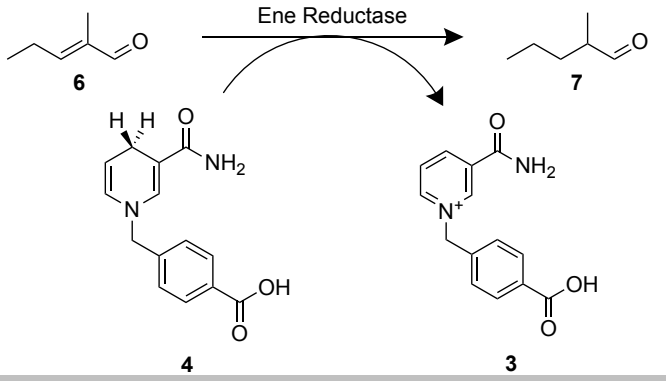
Stability of the mimics were also assessed (see Figure S44-45) as it has been reported the stability of dihydropyridines varies under different conditions.[45b] Compound 4 has been assessed in 3 different temperatures, 20, 30, and 40 ^{o}C, at 5 different pH each. From this study we have concluded that compound 4, similar to other dihydropyridine compounds, are stable in all temperatures in slightly basic to basic conditions. In acidic, weakly acidic, and neutral conditions, the compound had a fast rate of dissociation. Therefore, the studies were performed and reported at pH 7.5 or greater in order to ensure the compound was stable through all tests while still maintaining a physiological pH for the enzyme to functionally operate at.

Crystallographic Studies

Single crystals suitable for X-ray crystallography analysis were obtained for compounds **1** and **2.** Compound **1** crystallized from dimethyl sulfoxide in the trigonal space group $P3_1$, while crystals from compound **2** were obtained by slow evaporation from chloroform and diethyl ether and crystallized in the triclinic space group P-1. Details of the crystallographic analyses are provided in Tables **S1** - **S4**. The molecular structures for compounds **1** and **2** are shown in Figure 1a and b. Compound **1** exhibits a single hydrogen bonding interaction between the amide nitrogen and the bromide anion (d[N1-Br1] = 3.524(5) Å). Hydrogen bonding involving amide NH and Br anions were also reported for a similar nicotinamide conjugate, N-(3-(aden-9-yl)propyl)-3-carbamoylpyridinium bromide, with a distance of 3.397(7) Å.[46] In contrast, compound **2** engages in three intermolecular hydrogen bonding interactions resulting in the formation of a supramolecular assembly involving the amide and carbonyl groups of adjacent molecules, and a linkage through a bromide anion to neighboring molecules with distances of d[N1—O1] = 2.870(3) Å, d[N2-Br1] = 3.356(2) Å, and d[O4—Br1] = 3.1811(19) Å. Similar to the molecular structure of nicotinamide,[47] N-benzyl nicotinamide, [48] a very commonly reported NAD^+ mimic, and the natural NAD^+ itself,[49–51] the nicotinamide moiety of compound **2** engages in intermolecular bonding between neighboring nicotinamide moieties through the carbonyl and amide nitrogen functional groups. See figure 1c. The hydrogen bonding distances between the amide and carbonyl groups for the nicotinamide compounds stated above are reported to be in the range of 2.87-2.99 Å, which compound **2** also falls into (2.870(3) Å). In addition, compound **2** crystalized in the triclinic space group similar to the crystal structure of NAD^+ and as expected the pyridine group remains planar.[48] A more detailed look of the intermolecular patterns between many molecules of compound **2** can be seen in Figure **S33**. Next, the ability of the conjugates to be used as NADH mimics that enable biochemical hydride transfer reactions was probed.

Biochemical Evaluation of Nicotinamide Derivatives

The nicotinamide conjugates were biochemically evaluated in the enzyme ER, (EC 1.3.1.16) to explore the ability of the compounds to act as a coenzyme mimic of NADH. ER catalyzed reactions in the absence of NADH analogs, served as negative controls and thus it was decided to investigate these reactions further employing our synthetic nicotinamide derivatives as a NADH analogue. In addition, research into NADH-dependent biocatalytic alkene reduction catalyzed by these enzymes have been described in respect to their applications in industrial processes.[52] ER also exhibits cofactor promiscuity and was reported to accept a wide range of substrates which emphasizes the catalytic versatility and the expansion of ER use.[52] Therefore, the NADH analogues were then evaluated in ER where clear controls were run prior and precise results were drawn. Three substrates were chosen in this study due to their wide use but lack of kinetic data reported in ER. Due to solubility issues of compound **5,** it was not possible to carry out an accurate measurement of enzyme activity. In addition, based on the bulkiness of the amino acid substituent on the amide nitrogen, a proper fit in the active site may not be possible similar to the Hantzsch ester previously tested. However, compound **4** acts as a coenzyme mimic in the active enzyme and enzyme kinetics were performed for the enzymatic Ene reductions (see Table 2). These reductions were conveniently followed by GC-MS analysis, allowing the identification of the reduced product. This data is presented in Figures **S26-28** (compound **4**) and **S32-34** (NADH), and the retention times for the substrates and products are reported in Table **S6**. In addition, the enzymatic transformations were followed spectroscopically by monitoring the characteristic absorbance of the dihydropyridine derivative **4** at λ_{max} = 360 nm. A decrease at 360 nm is the result of substrate conversion. Scheme 2 shows the reduction of the C=C bond in the substrate 2-methyl-2-pentenal in ER using compound **4** as the coenzyme. Firstly, we were able to calculate the conversion of compound **4** into compound **3** using the calculated molar extinction coefficient, reported in Table **S5**, and the absorbance values at beginning of



the reaction and after 6 h. The highest conversion of compound **4** to compound **3** was found with the substrate 2-methyl-2-pentenal (**6**), with a conversion of 93%. The conversion when using the other two substrates with ER was not nearly as high with values of 31% for carvone (**8**) and 25% with the ketoisophorone (**10**).

Scheme 2: Scheme showing the enzymatic transformation in Ene Reductase of compound **6** to compound **7** using compound **4** as the coenzyme.

Secondly, we were able to confirm that compound **4** was capable of functionally substituting the natural coenzyme NADH in the enzymatic reaction in ER. Like NAD^+/NADH, compounds **3** and **4** exhibit absorbance's at 280 nm for the oxidized compounds and the additional peak at 340-360 nm for the reduced compounds. We were able to monitor the enzymatic reaction by observing the decrease in absorbance at 360 nm further proving that compound **4** can substitute NADH in the reductase enzyme. Figure 2 shows the decrease in the absorbance at 360 nm for compound **4** (a) and for NADH (b) over a 6-hour time period at times 60 s, 4500 s, 9000 s, 14400 s, and 21600 s. Figure 2c and d shows the reaction profile of the enzymatic reaction with compound **4** and NADH over time, and clearly shows the decrease in absorbance from the start of the reaction to the end of the 6-hour time course. Through both GC-MS and UV studies it is concluded that all three products of the enzymatic reaction were detected and the decrease in absorbance corresponding to substrate conversion was monitored. Next the kinetics of the enzymatic transformation was explored with compound **4** and the three substrates.

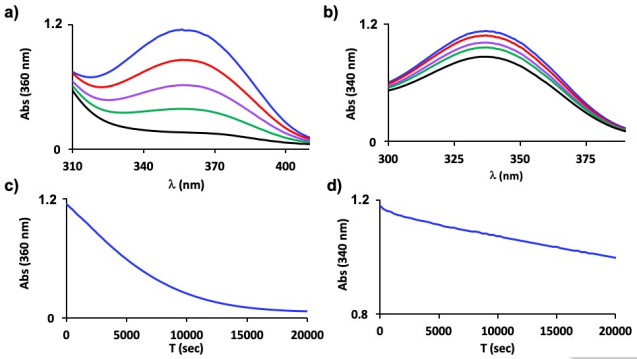


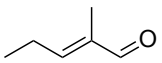
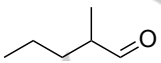
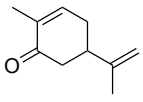
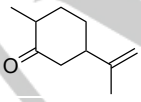
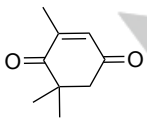
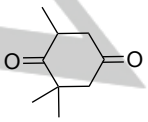
Figure 2: A) UV/vis spectra showing the decrease in absorbance at 360 nm with compound **4** as the coenzyme in 50 mM MOPS buffer + 5 mM $CaCl_2$ pH 7.5 containing 0.002umol of ER with 0.67 mM 2-methyl-2-pentenal as the substrate at 30 °C. B) UV/vis spectra showing the decrease in absorbance at 360 nm with NADH as the coenzyme in 50 mM MOPS buffer + 5 mM $CaCl_2$ pH 7.5 containing 0.002umol of ER with 0.67 mM 2-methyl-2-pentenal as the substrate at 30 °C. C) Absorbance recorded at T=21600 s (black), T= 14400 s (green), T= 9000 s (purple), T= 4500 s (red), T= 60s (blue). B) Reaction profile of the enzymatic C=C reduction using compound **4** as the coenzyme and 2-methyl-2-pentenal as the substrate over 6 hours. D) Absorbance recorded at T=21600 s (black), T= 14400 s (green), T= 9000 s (purple), T= 4500 s (red), T= 60s (blue). B) Reaction profile of the enzymatic C=C reduction using NADH as the coenzyme and 2-methyl-2-pentenal as the substrate over 6 hours.

Kinetic Evaluation of Compound 4 as a NADH Mimic

Michaelis-Menten kinetics were evaluated where the enzyme and coenzyme mimic concentrations remained constant while the substrate concentrations were varied. Experiments were performed with five different concentrations, 0.16, 0.33, 0.67, 1.67, and 3.33 mM for each of the three substrates, 2-methyl-2-pentenal **(4),** carvone **(6)**, and ketoisophorone **(8)**. Each reaction was monitored over 6 hours and readings were taken in 5 minute intervals. This was repeated with the natural NADH compound for a side by side comparison. All raw kinetic data is provided in the Supplemental Information section in Figures **S23-34.** From this, Michaelis-Menten curves and Lineweaver-Burk plots were constructed for each substrate (Figure **S33-43)** in order to determine the K_m and V_{max} values. See Table 2. In addition, the catalytic efficiencies (K_m/k_{cat}) were calculated and reported in Table 2 using the V_{max} values.

From the side by side comparison of our compound **4** and NADH, we now provide a second example of a coenzyme mimic outperforming the natural NADH coenzyme. In addition, we provide kinetic data for a Class II type ER and show its acceptance to a nicotinamide biomimetic and to a range of substrates. As shown in Figure 2, in over 6 hours, compound **4**

Table 2: Table listing the substrate and products used in ER with compound **4** and NADH as the coenzyme and the reported experimental Michaelis-Menten constant, K_m, (mM), V_{max} values mU) and turnover number, k_{cat}, (mM/min) found for each substrate.

Substrate	Product	K_m (compound 4)	V_{max} (compound 4)	k_{cat}/K_m (compound 4)	K_m (NADH)	V_{max} (NADH)	k_{cat}/K_m (NADH)
 (6)	 (7)	0.07 ± 0.016	15 ± 0.04	111.36	0.03 ± 0.0094	0.8 ± 0.05	11.62
 (8)	 (9)	0.22 ± 0.067	4 ± 0.6	7.97	0.36 ± 0.0503	0.9 ± 0.04	1.22
 (10)	 (11)	0.26 ± 0.046	3 ± 0.07	5.32	0.48 ± 0.299	0.6 ± 0.04	0.61

shows a decrease of the peak at 360 nm to an absorbance of almost 0. While NADH also shows a decrease, it is not nearly as fast as the turnover of compound **4** over the 6 hour period. When comparing the catalytic constants, it further proves that compound **4** outperformed NADH as a higher V_{max} and catalytic efficiency was reported for each substrate (Table 2)**.** For both compound **4** and NADH, a preference for substrate **6** is found in the enzyme over the other two substrates **8** and **10** as a lower K_m value was reported and a higher conversion. The K_m for substrate **6** was 0.077 mM and the V_{max} was 0.0147 µmol/min while for substrates **8** and **10**, which has industrial relevance due to the importance of the product, levodione **11** in carotenoids synthesis,[40] were 0.175 mM and 0.295 mM with a V_{max} value of 0.0033 and 0.0028 µmol/min, respectively. These values are in a similar range of other reported substrates used in ER transformations.[53][54] From this data, it can be concluded that conjugate **4** is an effective NADH mimic in the three reactions examined here allowing the transformation of substrates **6**, **8**, and **10** into their corresponding products.

Conclusions

Due to the expanding interest in exploring nicotinamide derivatives as mimics for biochemical transformations involving NADH and NAD^+, three novel conjugates were synthesized and their chemical properties, including their redox behavior were examined as potential mimics for the natural redox agents. It was possible to reduce compounds **1** and **3** into their corresponding dihydropyridines, **4** and **5**. The reduced compounds were shown to exhibit properties of typical dihydropyridine systems including displaying the characteristic absorbance at 360 nm. Compound **4** was exploited in biochemical transformations involving the enzyme Ene reductase, which allowed the enzymatic reduction of three unsaturated substrates into their corresponding products, and was proved to act as a NADH mimic. In addition, compound **4** outperformed the natural coenzyme having a higher catalytic efficiency and V_{max} value providing the second example of a nicotinamide biomimetic that outperforms the natural NADH in ER. Kinetic data is reported for the biotransformation with three substrates **6**, **8**, and **10** and each were shown to have comparable, if not better, catalytic values to the natural NADH. Both NADH and compound **4** performed best with substrate **6**, having the lowest K_m value of the three substrates and a low value compared to other similar substrates reported in ER in the literature.[34,53,54] Here, we also provide kinetic data for substrates that have been used in ER but lack the kinetic values, as well as kinetics for a class 2 ER which is not heavily reported compared to classes 1 and 3. Conjugate **4** is able to take on the role of a NADH mimic, possessing comparable kinetics in ER catalyzed biochemical transformations, suggesting that there is potential of **4** to be use useful in other enzymatic reactions that use NADH as the coenzyme.

Experimental Section

General Considerations

Nicotinamide, nicotinic acid, n-hydroxysuccinamide, pyridine, sodium dithionite, 2-methyl-2-pentenal, carvone, ketoisophorone, DCC, 1,2-dimethoxyethane, 4-bromomethyl benzoic acid, 5-bromo valeric acid, and Ene Reductase were all purchased from Sigma-Aldrich. Boc-Phe-OMe was purchased through Advanced ChemTech. All reagents were used without further modifications unless specified. Milli-Q water was used throughout this study for all purposes including electrochemistry, sample solutions and rinsing.

Synthesis of Compound 1

Compound **1** was prepared by synthesizing a nicotinic acid ester. N-hydroxysuccinamide (35 mmol) and DCC (35 mmol) were added into a solution of nicotinic acid (35 mmol) dissolved in 70 mL of pyridine. The solution was stirred 20 hours then filtered, re-dissolved in chloroform and washed 3 times with water. The crude product was then recrystallized with ethanol to give white needles.

The ester was then dissolved in 1,2-dimethoxyethane and cooled in an ice bath. A sodium bicarbonate solution containing 16 mmol of the amino acid phenylalanine was added to the ester solution drop wise over half an hour. The solution was left to stir for 2 hours at 0 ^oC then at room temperature for 14 hours. The solution was then filtered and acidified with 10% citric acid to a pH of 4 and then washed with ethyl acetate. The organic layer was dried with sodium sulfate and evaporated under pressure. The oil product was purified by silica column chromatography using dichloromethane and methanol as eluents.

7 mmol of the nicotinoyl-L-phenylalanine compound was dissolved in 50 mL of 1,4-doixane/ 12.5 mL methanol solution. 15 mmol of benzyl bromide was added to this solution and was refluxed for 5 hours in an oil bath. The product was filtered and washed with hexanes to obtain the final pure product.

1H NMR (500 MHz, chloroform-d) 10.53 (s, 1H, pyr-1), 9.73 (d, 1H, amide NH), 8.91 (d, 1H, pyr-5), 8.84 (d, 1H, pyr-3), 7.97 (m, 2H, pyr-4), 7.62 (dd 2H, aromatic C-H), 7.49 (m, 5H, aromatic C-H), 7.26 (t, 2H, aromatic C-H), 7.18 (t, 1H, aromatic C-H), 6.11 (s, 2H, CH_2), 4.94 (m, 1H, CHα), 3.75 (s, 3H, CH_3), 3.54(dd, 1H, CH_2β), 3.44 (dd, 1H, CH_2β). 13C NMR (DMSO-d6): δ 171.76 (1C of COOMe), δ 161.81 (1C of CONH), δ 145.24 (3C, pyr-1,3,5), δ 137.45 (2C, pyr-2 & aromatic C), δ 133.98 (2C, aromatic C and pyr-4), δ 129.34 (10C, aromatic C), δ 64,10 (1C, CH_2), δ 55.08 (1C, α C), δ 52.70 (1C, CH_3), δ 36.76 (2C, β C), Expected MS: m/z 375.1703 [M +], Experimental MS: m/z 375.1704 $C_{23}H_{23}N_2O_3^+$ Yield: 76%

Synthesis of Compound 2

15 mmol of nicotinamide was dissolved in a 50 mL 1,4-dioxane/25 mL methanol mixture. 7 mmol of 5-bromo valeric acid was added and the solution was refluxed for 6 hours. The solution was filtered and the solid was washed with dichloromethane and hexanes to obtain the pure product.

1H NMR (500 MHz, DMSO-d6) 12.14 (s, 1H, COOH), 9.51 (m, 1H, pyr-1), 9.22 (d, 1H, pyr-5), 8.94 (d, 1H, pyr-3), 8.57(s, 1H, NH_2), 8.28 (dd, 1H, pyr-4), 8.19 (s, 1H, NH_2), 4.67(t, 2H, CH_2), 2.29 (t, 2H, CH_2), 1.97 (m, 2H, CH_2), 1.51 (p, 2H, CH_2), 13C NMR (DMSO-d6): δ 174.51(1C of COOH, δ 163.28 (1C of $CONH_2$), δ 146.81 (1C, pyr-3), δ 145.28 (1C, pyr-1), δ 143.65 (1C, pyr-5), δ 134.35 (1C, pyr-2), δ 128.33(1C, pyr-4), δ 61.28 (1C, CH_2), δ 33.29 (1C, CH_2), δ 30.54 (1C, CH_2), δ 21.33 (1C, CH_2) Experimental MS: m/z 223.1077 [M +], Expected MS: m/z 223.1074[M +], $C_{11}H_{15}N_2O_3^+$ Yield: 67%

Synthesis of Compound 3

15 mmol of nicotinamide was dissolved in a 50 mL 1,4-dioxane/25 mL methanol mixture. 7 mmol of 4-bromomethyl benzoic acid was added and the solution was refluxed for 6 hours. The solution was filtered and the solid was washed with dichloromethane and hexanes to obtain the pure product.

1H NMR (500 MHz, DMSO-d6) 13.17 (s, 1H, COOH), 9.62 (s, 1H, pyr-1), 9.28 (d, 1H, pyr-5), 8.98 (d, 1H, pyr-3), 8.59 (s, 1H, NH_2), 8.31 (dd, 2H, pyr-4), 8.19 (s, 1H, NH_2), 8.00 (d, 2H, aromatic C-H), 7.63 (d, 2H, aromatic C-H), 6.00 (s, 2H, CH_2), 13C NMR (DMSO-d6): δ 167.20 (1C of COOH), δ 163.16 (1C of $CONH_2$), δ 147.05 (1C, pyr-5), δ 138.93 (1C, aromatic C), δ 134.68 (2C, pyr-2), δ 132.08 (1C, pyr-4), δ 130.47 (2C, aromatic C), δ 129.48 (2C, aromatic C), δ 128.83 (1C, aromatic C), δ 63.51 (1C, CH_2), Expected MS: m/z 257.0921 [M +], Experimental MS: m/z 257.0918 $C_{14}H_{13}N_2O^+$ 78%

Synthesis of Compound 4

6 mmol of sodium bicarbonate was added to 20 mL of Mili-Q water. Once dissolved 1 mmol of compound **3** was added and dissolved. Under N_2 and in the dark, 4 mmol of sodium dithionite was added slowly to the mixture. The reaction was stirred vigorously for 3 hours at room temperature. The pure compound was precipitated out using concentrated HCl.

1H NMR (500 MHz, DMSO-d6) 13.16 (s, 1H, COOH), 7.92 (d, 2H, aromatic CH), 7.36 (d, 2H, aromatic CH), 6.99 (s, 1H, pyr-1), 6.58 (s, 2H, NH_2), 5.94 (d 1H, pyr-5), 4.63 (dt 1H, pyr-4), 4.39 (s, 2H, CH_2), 2.97 (d, 2H, pyr-3), 13C NMR (DMSO-d6): δ 169.44 (1C of COOH), δ 167.69 (1C of $CONH_2$), δ 143.72 (3C, pyr-1), δ 138.26 (1C, aromatic C), δ 130.07 (2C, aromatic C), δ 128.77 (1C, pyr-5), δ 127.83 (1C, aromatic C) δ 127.60 (1C, aromatic C), δ 102.44 (1C, pyr-2), δ 101.14 (2C, pyr-4), δ 55.97 (2C, CH_2) δ 22.76 (1C, pyr-3) Expected for MS: m/z 259.1004 [M +H+], Experimental MS: m/z 259.1069 $C_{14}H_{14}N_2O_3$ Yield: 57%

Synthesis of Compound 5

6 mmol of sodium bicarbonate was added to 20 mL of Mili-Q water. Once dissolved 1 mmol of compound **1** was added along with 15 mL of dichloromethane until fully dissolved. Under N_2 and in the dark, 4 mmol of sodium dithionite was added slowly to the mixture. The reaction was stirred vigorously for 3 hours at room temperature. The compound was collected in the organic layer and was purified through silica column chromatography.

1H NMR (500 MHz, chloroform-d) 8.68 (s, 1H, amide NH), 7.14-7.48 (m, 10H, aromatic CH), 5.73 (dd, 1H, pyr-1), 4.99 (dq, 1H, pyr-5), 4.49 (d, 1H, CHα), 4.41 (dd, 1H, pyr-4), 4.30 (q, 2H, CH_2), 3.73 (s, 3H, CH_3), 3.34 (d, 2H, pyr-2), 3.15 (m, 2H, CH_2β). 13C NMR (chloroform-d6): δ 172.77 (1C of COOMe), δ 167.47 (1C of CONH), δ 141.39 (1C, pyr-1), δ 137.48 (1C, aromatic C), δ 136.24 (1C, pyr-5), δ 128.24 (10C, aromatic C), δ 99.77 (1C, pyr-2), δ 98.66 (1C, pyr4), δ 84.52 (1C, CH_2), δ 57.81 (1C, CHα), δ 54.95 (1C, CH_3), δ 52.83 (1C, CH_2β), δ 38.27 (1C, pyr-2). Expected MS: m/z 377.1787 [M +H+], Experimental MS: m/z 377.1864 $C_{23}H_{24}N_2O_3$ Yield: 37%

UV/vis studies of the Enzymatic Transformations with compound 4

In a quartz cuvette, 100 ug/mL of Ene Reductase, 0.1 mM of compound **4**, 3 mL of MOPS buffer, and varying concentrations of each substrate from 0.16 mM- 3.33 mM was added. The cell was kept at 30 °C. The decrease of the absorbance at 360 nm which is characteristic of the dihydropyridine was monitored with an 8453 w Peltier UV/vis spectrophotometer.

GC-MS Studies

GC-MS studies were carried out on an Agilent DC-624 (30m x 0.32 (1.0 µm df)). The enzymatic reactions were set-up in a temperature-controlled water bath. The buffer (MOPS, 50 mM, pH 7.5 with 5 mM $CaCl_2$), substrate (2-methyl-2-pentenal, carvone, and ketoisophorone), coenzyme mimic (**4**) and enzyme (ER) were added in this order. 250 uL were taken at different time intervals over 6 hours and extracted with ethyl acetate (600 uL) and centrifuged for 2 minutes. The ethyl acetate layer was then analyzed by GC-MS.

Nuclear Magnetic Resonance (NMR) Studies

Compounds **1-3** were characterized using NMR spectroscopy (Bruker spectrometer 500 MHz). Compound **1** and **5** was dissolved in chloroform-d while compound **2, 3** and **4,** was dissolved in dimethoxy-sulfoxide-d6 all at a concentration of 2 mM. Spectra are attached in the Supplemental Information section.

Electrochemical Studies

The electrochemical experiments were carried out at room temperature using a CHI660B electrochemical workstation (CH Instruments Inc). All electrochemical experiments used a glassy carbon working electrode, glassy carbon as a counter electrode, and Ag/AgCl (saturated with 3M KCl) reference electrode. The glassy carbon electrodes were polished with 0.05 µM Al_2O_3, then sonicated in Milli-Q water, ethanol, and Milli-Q water again for 10 minutes each to fully remove any absorbed Al_2O_3. The electrodes were thoroughly rinsed with Milli-Q water after each sonication. Both electrodes were electrochemically cleaned using a 1 mM KOH solution. 1 mM solutions of compounds **1-3** were prepared in 1 mM phosphate buffer pH 7.05 and were used for all electrochemical experiments. Cyclic voltammetry (CV) was performed at a 100 mV/s scan rate.

X-ray Crystallography Studies

Crystallographic data were collected on a Bruker Kappa APEX-DUO diffractometer using a Copper ImuS tube with multilayer optics. The data were processed using APEX2 and SAINT. Absorption corrections were carried out using SADABS. The structure was solved with SHELXT and refined using SHELXL-2014 for full-matrix least-squares refinement that was based on F2. All H atoms were included in calculated positions and allowed to refine in riding-motion approximation with U ~ iso ~ tied to the carrier atom

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Keywords: nicotinamide• redox • NADH mimics • enzyme kinetics • biocatalysis

Acknowledgements

[1] H. Han, Z. He, J. Liu, Y. Chen, S. Liu, *Ionics.* **2015**, *21*, 167–174.

[2] S. Maurya, S. Shin, K. Sung, S. Moon, *J. Power Sources* **2014**, *255*, 325–334.

[3] K. Eto, Y. Tsubamoto, Y. Terauchi, T. Sugiyama, T. Kishimoto, N. Takahashi, N. Yamaucvhi, N. Kubota, S. Murayama, T. Aizawa, et al., *Science.* **1999**, *283*, 981–986.

[4] F. P. Guengerich, W. R. Brian, M. Iwasaki, M. Sari, C. Báárnhielm, P. Berntason, *J. Med. Chem.* **1991**, *34*, 1838–1844.

[5] N. M. Evdokimov, I. V Magedov, A. S. Kireev, A. Kornienko, *Org. Lett.* **2006**, *8*, 899–902.

[6] P. Radiolysis. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **1968**, *162*, 327–337.

[7] J. W. Foster, A. G. Moat, *Microbiol. Rev.* **1980**, *44*, 83–105.

[8] C. Canto, K. Menzies, J. Auwerx, *Cell Metab.* **2015**, *22*, 31–53.

[9] R. L. Reyes, K. Tanaka, *KIMIKA* **2017**, *28*, 32–43.

[10] Q. Chen, K. Gao, Y. Duan, Z. Ye, L. Shi, Y. Yang, Y. Zhou, *J. Am. Chem. Soc.* **2012**, *134*, 2442–2448.

[11] S. G. Kim, S. Oka, Y. Ohnishi, *Bioorg. Chem.* **1977**, *29*, 21–29.

[12] I. Ali, A. Gill, S. Omanovic, *Chem. Eng. J.* **2012**, *188*, 173–180.

[13] I. Ali, T. Khan, S. Omanovic, *J. Mol. Catal. A: Chem.* **2014**, *387*, 86–91.

[14] R. Saito, S. Naruse, K. Takano, K. Fukuda, A. Katoh, Y. Inoue, *Org. Lett.* **2006**, *8*, 2067–2070.

[15] M. Beley, J. Collin, *J. Mol. Catal.* **1993**, *79*, 133–140.

[16] C. S. Sevov, R. E. M. Brooner, E. Chenard, R. S. Assary, J. S. Moore, J. Rodriguez-Lopez, M. S. Sanford, *J. Am. Chem. Soc.* **2015**, *4*, 14465–14472.

[17] A. McSkimming, S. B. Colbran, *Chem. Soc. Rev.* **2013**, *42*, 5439–5488.

[18] C. Lim, A. M. Holder, J. T. Hynes, C. B. Musgrave, *J. Am. Chem. Soc.* **2014**, *136*, 16081–16095.

[19] N. R. Robles, F. Fici, G. Grassi, *Hypertens. Res.* **2017**, *40*, 21–28.

[20] X. C. Tang, M. J. Pikal, L. S. Taylor, *Pharm. Res.* **2002**, *19*, 477–483.

[21] J. Zhao, N. X. Wang, W. W. Wang, G. X. Wang, Y. H. Liu, L. Li, J. L. Yu, X. L. Tang, *Molecules* **2007**, *12*, 979–987.

[22] Y. Okamoto, V. Köhler, C. E. Paul, F. Hollmann, T. R. Ward, *ACS Catal.* **2016**, *6*, 3553–3557.

[23] C. Nowak, A. Pick, P. Lommes, V. Sieber, *ACS Catal.* **2017**, *7*, 5202–5208.

[24] C. Nowak, B. Beer, A. Pick, T. Roth, P. Lommes, V. Sieber, *Front. Microbiol.* **2015**, *6*, 1–9.

[25] A. Geddes, C. E. Paul, S. Hay, F. Hollmann, N. S. Scrutton, *J. Am. Chem. Soc.* **2016**, *138*, 11089–11092.

[26] R. A. Sheldon, P. C. Pereira, *Chem. Soc. Rev.* **2017**, *46*, 2678–2691.

[27] G. Li, J. bo Wang, M. T. Reetz, *Bioorganic Med. Chem.* **2018**, *26*, 1241–1251.

[28] R. J. Ansell, D. A. P. Small, C. R. Lowe, *J. Mol. Catal. B Enz* **1997**, *3*, 239–252.

[29] H. C. Lo, R. H. Fish, *Angew. Chem. Int. Ed.* **2002**, *41*, 478–481.

[30] H. C. Lo, O. Buriez, J. B. Kerr, R. H. Fish, *Angew. Chem. Int. Ed* **1999**, *38*, 1429–1432.

[31] H. C. Lo, C. Leiva, O. Buriez, J. B. Kerr, M. M. Olmstead, R. H. Fish, *Inorg. Chem.* **2001**, *40*, 6705–6716.

[32] J. Lutz, F. Hollmann, T. V. Ho, A. Schnyder, R. H. Fish, A. Schmid, *J. Organomet. Chem.* **2004**, *689*, 4783–4790.

[33] H. C. Lo, J. D. Ryan, J. B. Kerr, D. S. Clark, R. H. Fish, *J. Organomet. Chem.* **2017**, *839*, 38–52.

[34] T. Knaus, C. E. Paul, C. W. Levy, S. De Vries, F. G. Mutti, F. Hollmann, N. S. Scrutton, *J. Am. Chem. Soc.* **2016**, *138*, 1033–1039.

[35] C. E. Paul, I. W. C. E. Arends, F. Hollmann, *ACS Catal.* **2014**, *4*, 788–797.

[36] J. D. Ryan, R. H. Fish, D. S. Clark, *ChemBioChem* **2008**, *9*, 2579–2582.

[37] C. E. Paul, S. Gargiulo, D. J. Opperman, Lavandera, I. Gotor-Fernandez, V. Gotor, V. Taglieber, A. Arends, I. W. C. E. Hollmann, F. *Org. Lett.* **2013**, *15*, 180–183.

[38] H. S. Toogood, T. Knaus, N. S. Scrutton, *ChemCatChem* **2014**, *6*, 951–954.

[39] H. Sedlmaier, W. Tischer, P. Rauschenbach, H. Simon, *FEBS Lett.* **1979**, *100*, 129–132.

[40] R. Stuermer, B. Hauer, M. Hall, K. Faber, *Curr. Opin. Chem. Biol.* **2007**, *11*, 203–213.

[41] K. Tauber, M. Hall, W. Kroutil, W. M. F. Fabian, K. Faber, S. M. Glueck, *Biotechnol. Bioeng.* **2011**, *108*, 1462–1467.

[42] a) A. Scholtissek, D. Tischler, A. Westphal, W. van Berkel, C. Paul, *Catalysts* **2017**, *7*, 130. b) V. Carelli, F. Liberatore, L. Scipione, B. Di Rienzo, S. Tortorella, *Tetrahedron,* **2005,** *61,* 10331-10337. c) Y-S. Wong, C. Marazano, D. Gnecco, B. C. Das, *Tetrahedron. Lett.* **1994,** *35,* 707-710. d) E. M. P. Silva, P. A. M. M. Varandas, A. M. S. Silva, *Synthesis,* **2013,** *45,* 000A-00AK.

[43] E. Hald, P. Lehmann, J. Zeigenhorn, *Clin. Chem.* **1975**, *21*, 884–887.

[44] T. Quinto, V. Köhler, T. R. Ward, *Top. Catal.* **2014**, *57*, 321–331.

[45] a) S. Kochius, J. B. Park, C. Ley, P. Könst, F. Hollmann, J. Schrader, D. Holtmann, *J. Mol. Catal. B Enz* **2014**, *103*, 94–99. b) C. Nowak, A. Pick, L-I. Csepei, V. Sieber, *ChemBioChem,* **2017,** *18,* 1944-1949.

[46] P. L. Johnson, J. K. Frank, I. C. Paul, *J. Am. Chem. Soc* **1973**, *95*, 5377–5385.

[47] W. B. Wright, G. S. D. King, *Acta Cryst.* **1954**, *7*, 283–288.

[48] I. L. Karle, *Acta Cryst.* **1961**, *14*, 497–502.

[49] B. Guillot, C. Jelsch, C. Lecomte, *Acta Cryst.* **2000**, *C56*, 726–728.

[50] B. Guillot, C. Lecomte, A. Cousson, C. Scherf, C. Jelsch, *Acta Cryst.* **2001**, *D57*, 981–989.

[51] B. S. Reddy, W. Saenger, K. Mühlegger, G. Weimann, *J. Am. Chem. Soc* **1981**, *103*, 907–914.

[52] H. S. Toogood, N. S. Scrutton, *ACS Catal.* **2018**, *8*, 3532–3549.

[53] H. Simon, J. Bader, H. Gunther, S. Neumann, J. Thomas, *Angew. Chem. Int. Ed.* **1985**, *24*, 539–553.

[54] R. M. D. Verhaert, B. Tyrakowska, R. Hilhorst, T. Schaafsma, C. Veeger, *Eur. J. Biochem.* **1990**, *187*, 73–79.

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Layout 1:

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In the present study three nicotinamide-containing conjugates are synthesized, and biochemically evaluated in ER. Two molecular structures were obtained by X-ray crystallography of compounds **1** and **2**. Compound **4** was biochemically evaluated and it was found that it can functionally substitute and outperform the natural coenzyme NADH with comparable kinetic values for three substrates.

*Natashya Falcone, Zhe She, Jebriel Syed, Alan Lough, Heinz-Bernhard Kraatz**

1 – 8

Synthesis, and Biochemical Evaluation of Nicotinamide Derivatives as NADH Analogues in Ene Reductase

Layout 2:

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