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Bioinspired design of a hybrid bifunctional enzymatic/organic electrocatalyst for site selective oxidation of aliphatic alcohols

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Materials and Methods:

Chemicals:

Tris base, Tris hydrochloride, hydrochloric acid, sodium chloride, 10X phosphate buffered saline, sodium phosphate monobasic, sodium phosphate dibasic, sodium azide, sodium dodecyl sulfate, (±)-epichlorohydrin, tetrabutylammonium hydrogen sulfate, sodium hydroxide, ethyl acetate, calcium chloride, 230-400 mesh silica gel, methylene chloride, methanol, acetonitrile, sulfuric acid butyraldehyde, and butanone were purchased from Fisher Scientific and used as received.

4-hydroxy-TEMPO, anhydrous magnesium sulfate, 1-butanol, 2-butanol, beta-nicotinamide dinucleotide sodium salt, glycine, o-phenylenediamine, sodium ascorbate, acrolein-2,4-dinitrophenylhydrazone, butyraldehyde-2,4-dinitrophenylhydrazone, butanone-2,4-dinitrophenylhydrazone, and phosphoric acid were purchased from Sigma-Aldrich and used as received. 2,4-dinitrophenylhydrazine was purchased from Sigma-Aldrich and recrystallized before use.

Synthesis:

AdhD expression and purification. Overexpression of AdhD was performed following a previously established protocol[1]. Purification was performed using a Bio-Rad Biologic DuoFlow 10 System with BioFrac Fraction Collector (Bio-Rad), equipped with a Maximizer valve system (Bio-Rad), QuadTec detector (Bio-Rad), 2 x AVR7-3 Automated Sample Injection Valves (Bio-Rad) plumbed for reverse flow chromatography, and a HiPrep DEAE FF 16/10 weak anion-exchange column (GE Healthcare). The buffers used were (A1) 50 mM Tris pH 8.0, (B1) 50 mM Tris, 2 M sodium chloride pH 8.0, (A2) water, and (B2) 2 M sodium chloride.

After column equilibration with A1 at 5 ml/min, lysate was injected from the sample loop with A1 at 1 ml/min. 4x the sample volume of A1:B1 95:5 at 2 ml/min was used to rinse the column. The direction of the mobile phase flow through the column was then reversed using the 2nd 7-port valve and a gradient from A1:B1 88:12 to A1:B1 85:15 over a volume 2.5x the sample volume was started at 2 ml/min. 15 ml into the gradient, the fraction collector began collecting 5 ml fractions until the gradient ended. The column was then washed with A2:B2 50:50 for 2.5 column volumes at 5 ml/min, and all salt removed with A2 for 5 column volumes at 5 ml/min

Fractions were analyzed by SDS-PAGE using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell and AnyKD Mini-PROTEAN TGX Stain-Free Precast Protein Gels (Bio-Rad), those containing AdhD of sufficient purity were pooled and concentrated and the buffer was switched to PBS using Amicon 10kDa MWCO centrifugal filters (Fisher Sci). Enzyme concentration was determined through A_{280} measurements made using a Nano-Drop 2000C (Thermo Fisher)A280 function with the percent solution extinction coefficient (ε1%) set to 16.51 L g^{-1} cm^{-1}. Sodium azide was added to 0.2 wt% as an anti-microbial, and aliquots were stored at 4^{o}C.

4-glycidyl-TEMPO. 4-glycidyl-TEMPO was prepared following a protocol published by Song et. al[2]. 0.09 g tetrabutylammonium hydrogen sulfate and 2.69 g (±)-epichlorohydrin were dissolved in 10 mls of 50 wt% aqueous sodium hydroxide. 1 g 4-hydroxy-TEMPO was added to the mixture and left stirring at room temperature for 24 hours. The solution was poured into water and the product was extracted with ethyl

acetate. The organic fraction was then rinsed with brine, dried with $MgSO_4$ and $CaCl_2$, and the solvent was removed under reduced pressure. 4-glycidyl-TEMPO was isolated from this mixture by silica column chromatography using a burette packed with silica gel as the stationary phase, and mobile phases of methylene chloride and methanol respectively. 4-glycidyl-TEMPO was confirmed in the first fraction using H-NMR.

AdhD-TEMPO conjugation. 5 mg AdhD in PBS was transferred into micro centrifuge tubes and the volume adjusted to 800 µl with 1X PBS. The desired molar excess of 4-glycidyl-TEMPO was dissolved to a volume of 200 µl in PBS and added to the AdhD. The tubes were wrapped in foil and left on a shaker for 48 hours at room temperature. Unreacted 4-glycidyl-TEMPO was removed with a buffer exchange into fresh PBS using a HiTrap Desalting column with Sepharose (GE Healthcare) after the reaction mixtures were clarified by centrifugation at 15,000 x g for 10 minutes and filtration through regenerated cellulose syringe filters with 0.2 µm pores. 500 µl fractions were loaded into an FPLC sample loop and flow rate was set at 5 ml/min. After column equilibration, the sample was injected onto the column and the fraction collector began collecting 500 µl fractions. The fractions containing protein were identified by SDS-PAGE, pooled, and dialyzed against 2 liters of fresh PBS using 10 kD MWCO Snakeskin dialysis tubing (Fisher Sci) at 4° C overnight. The dialyzed solution was concentrated to desired storage concentration using 10 kD MWCO centrifuge filters. Concentration was determined using the same instrument and extinction coefficient as described above for AdhD. Sodium azide was not added for storage as it is electrochemically reactive under the conditions used in later analysis.

Chemical Assays:

Colorimetric assay for AdhD activity/selectivity. AdhD and AdhD-TEMPO oxidative activity towards 1- and 2-butanol was determined by measuring NADH generation across a range of substrate concentrations. Reactions were monitored in 100 µl volumes using round bottom 96-well plates. The reaction was initiated by adding substrate into wells to a final composition of 10 mM phosphate (pH 8.3), 137 mM sodium chloride, 80 nM AdhD or AdhD-TEMPO, and 750 µM NAD^+. A BioTek Synergy™ Hybrid Microplate Reader was used to monitor the absorbance of 340nm light. Initial reaction rates were determined using NADH's extinction coefficient (6200 M^{-1} cm^{-1}) and plotted against substrate concentration.

Cyclic voltammetry for 4-Glycidyl-TEMPO electrochemical activity/selectivity. Electrochemical measurements were performed using a VMP3 potentiostat (BioLogic USA) and 3-electrode electrochemical cells with 3mm glassy carbon disk working electrodes (ALS, CH Instruments), Ag/AgCl reference electrodes in either 1M KCl (CH Instruments), or 3M NaCl (ALS), and platinum wire counter electrodes. The buffer used for AdhD activity assays was also used as the buffered electrolyte, 137 mM sodium chloride buffered with 10 mM phosphate (pH 8.3). Voltage was cycled from 0 to 1 V at 5 mV/s.

Analysis of 4-glycidyl-TEMPO attachment to AdhD:

Native-PAGE: Bio-Rad's AnykD Mini-Protean TGX Stain-Free Precast Gels were used to run conjugated samples through Native-PAGE. 700 mls of electrophoresis buffer was made fresh by adding 2.1 g Tris-base (25 mM) and 10.08 g glycine (192 mM) to 500 mls of ddH_2O, then diluted to a final volume of 700 ml. A

TetraCell vertical electrophoresis system (Bio Rad) was used. Conjugated protein samples were mixed 1:2 with Native Sample Buffer (Bio-Rad) and immediately loaded into sample wells of the precast gels. The potential was set to 150V and the gels left to run for 60 minutes before the Stain-Free protein indicator was activated and imaged using a Bio-Rad ChemiDoc MP Imaging System.

Colorimetric assay to determine presence of TEMPO*. Solutions of 20mM ascorbic acid and 20 mM o-phenylenediamine were prepared in 150 mM phosphate buffer at pH 7.0, and wrapped in foil to keep them out of direct light. In 200 µl microcentrifuge tubes, 25 µl of the ascorbate solution was mixed with a 50 µl sample of conjugated protein using an 8-channel pipette and inverted several times. This was left to react for precisely 5 minutes, during which 25 µl of the OPD solution was placed into several wells of a 96-well microplate. After 5 minutes, the mixture was volumetrically transferred into the OPD containing wells. The plate was inserted into a BioTek Synergy™ Hybrid Microplate Reader and 340 nm light was monitored for 5 minutes.

The formation of dehydroxyascorbate (DHA)-quinoxaline is indicated by an increase in the absorbance of 340 nm light. This product was confirmed using ^{1}H-NMR, and compared to the purified product from a previously described procedure. Ascorbic acid (0.1 g, 0.57 mmol) was dissolved in a 3:1 mixture of methanol/water (3 mL), and added to a solution of benzoquinone (0.074 g, 0.68 mmol) in methanol (1 mL). The reaction mixture was stirred for 3 hours at room temperature. To the reaction mixture was added a solution of o-phenylenediamine (0.06 g, 0.57 mmol) in methanol (1 mL). The reaction mixture was stirred overnight at room temperature. Methanol was removed from the resulting solution under reduced pressure. DHA-quinoxaline was extracted from the aqueous solution using diethyl ether (3 times). The organic portions were combined and dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. ^{1}H-NMR (CD_3OD): 3.80-3.91 (2H, m), 4.37 (1H, m), 5.94 (1H d), 7.97-8.09 (2H, m), 8.24-8.32 (2H, m). See Figure S5.

Cyclic Voltammetry to measure electrochemical activity of conjugated AdhD-TEMPO. 100 µl of the conjugated samples were loaded into a 9mm OD glass vial (Bio-Logic USA) fitted with an ion permeable Vycor glass frit and placed into an SVC-2 electrochemical cell (Bio-Logic USA). A 3mm glassy carbon disk working electrode and 0.5 mm platinum wire counter electrode were placed in the sample. The sample vial and Ag/AgCl reference electrode were placed in the same electrolyte completing a circuit with the potentiostat. The voltage was cycled from 0V to 1V 3 times at each scan rate of 5, 20, and 100 mV/s.

Controls include buffer blanks, unmodified AdhD, 4-glycidyl-TEMPO, and samples obtained from the final filtrate from the final concentration step of the AdhD-TEMPO samples.

Proteomic LC-MS/MS. Samples of AdhD and AdhD-TEMPO were purified and submitted to the University of California, Riverside's IIGB Proteomics core. After acetone precipitation and trypsin digestion, peptide samples were separated with a nanoAcquity UPLC system (Waters Corp). A BEH130 C18 column with 1.7 µm particles and dimensions of 75 µm x 20 cm (Waters) was used as an analytical column. A Symmetry C18 column with 5 µm particles, measuring 180 µm x 20 mm (Waters) served as a trap/guard column for desalting. The solvent components for peptide separation were as follows: mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Sample desalting was completed with mobile phase A at flow rate of 20 ul/min for 15 min. The separation gradient for the

analytical column was as follows: at 0 to 1 min, 3% B; at 2 min, 8% B; at 50 min, 45% B; at 52 to 55 min, 85% B; at 56 to 70 min, 3% B. The nano-flow rate was set at 0.3 µl/min without flow-splitting.

The separated peptide fragments were introduced into an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher) by Electron Spray Ionization. The peptide ions were then fragmented by Collision Induced Dissociation. Data for all parent ions and second fragmentation ions was collected and run through the Mascot server for peptide identification using all possible peptide sequences that would result from AdhD cleavage by Trypsin. Search settings for post-translational modification were set to identify peptides that have an increase of 229 ± 1 da on histidine, cysteine, or lysine residues. Spectra for ions that Mascot indicated as matching that modification criteria were analyzed individually.

Analysis of AdhD-TEMPO oxidation activity and selectivity:

Carbonyls are not detectable by spectrophotometers and photo-diode arrays, nor did they have enough activity towards the stationary phase of the column in their natural state. All reaction samples had product yields quantified after derivatization by 2,4-DNPH.

DNPH recrystallization. Room temperature acetonitrile was saturated with 2,4-DNPH in a flask that was then covered and boiled. Acetonitrile was added if any solid 2,4-DNPH remained at boiling temperatures. After 2 hours, the temperature was reduced to 60^{o}C for another 2 hours. The flask was gently removed from the hot plate and allowed to reach room temperature. Once room temperature was reached and crystal growth was observed, the flask was transferred into a freezer for an hour, or until crystals stopped growing. The crystals were filtered from the saturated acetonitrile solution with a Buchner funnel and rinsed with cold acetonitrile. This recrystallization was repeated at least once, or as many times as necessary before the cold saturated acetonitrile the crystals were filtered from gave a clean chromatogram when run through an HPLC monitoring 360 nm wavelength light.

Cascade reaction setup. To determine the quantity of each oxidation product, the reaction conditions were set to activate both catalysts/active sites of the component catalysts. An electrochemical cell with 3 mm glassy carbon working electrode, coiled platinum wire counter electrode and Ag/AgCl reference electrode was used as a reactor. The reactions were run with 10 ml volumes and the final compositions were 10 mM phosphate, 137 mM NaCl, 125 mM each 1-, and 2-butanol, 500 µM NAD^+. AdhD and TEMPO, or AdhD-TEMPO was added last to initiate the reaction, and the potentiostat was set to maintain 800 mV vs SCE potential in the stirred cell. Samples were drawn from the reaction mixture at various time points and the relative concentration of the carbonyl products was determined by HPLC after derivatization with 2,4-DNPH. For controls, the reaction was set up with both individual catalysts, separately and together, with 1-, and 2-butanol, with and without NAD^+, and with and without electric potential applied (see Table S2).

Derivatization of carbonyls in time course samples. The EPAs method 8315A, Determination of carbonyl compounds by high performance liquid chromatography[4], was modified to eliminate the use of methylene chloride and to reduce the quantity of acetonitrile required, resulting in a more cost effective and environmentally friendly procedure.

The stationary phase of Bond Elut C18, 100mg 1ml solid phase extraction cartridges (Agilent) were used to immobilize 2,4-DNPH prior to the reaction being setup. The cartridges were placed in a vacuum manifold. Once the cartridges were wetted, they were not allowed to run dry until the immobilization was completed. First, cartridges were rinsed with 500 µl acetonitrile, then 500 µl of a solution of 10% acetonitrile in 0.1% phosphoric acid in water. 2 volumes of 1 ml of a solution of 10% acetonitrile in 0.1% phosphoric acid in water saturated with recrystallized 2,3-DNPH was then added to the cartridges, after which they were left under the vacuum for several minutes to dry. All cartridges were prepared in this manner with freshly prepared solutions no more than an hour before used for derivatizing reaction samples.

A sample derivatization solution was prepared at 1.5x. 300 µl of this solution was aliquoted into 1.5 ml microcentrifuge tubes, which would have 200 µl samples added and contain a final composition of 10 wt% acetonitrile and 0.4 µg of acrolein-2,4-DNPH used as the internal standard. This was to give ratio of 1 µg internal standard per ml of reaction sample.

Samples were drawn from the reaction in excess of 200 µl using a syringe, dispensed into a microcentrifuge tube containing 2 µl phosphoric acid to deactivate AdhD. 200 µl was transferred to a microcentrifuge tube containing 300 µl of the derivatization solution as described above. This 500 µl solution was mixed by inversion, then transferred to the 2,4-DNPH coated SPE cartridges. Positive pressure was applied to the top of the cartridge until liquid broke through the stationary phase, then the cartridge was left for the sample solution to flow under gravity, with the eluent being collected and reapplied to the cartridge twice to increase the time carbonyls were in contact with 2,4-DNPH.

After all samples had been drawn and put through derivatization cartridges for an appropriate amount of time, the cartridges had any excess sample pushed through with positive pressure, before they were placed into the vacuum manifold again. A stepwise elution was performed to minimize the amount of unreacted DNPH in each sample. 500 µl of 10% acetonitrile in 0.1% phosphoric acid in water was followed by 3 x 500 µl of 30% acetonitrile in water, then the cartridges were left under vacuum to dry for several minutes. The derivatized analytes of interest were eluted from the cartridge into 1.5 ml microcentrifuge tubes using 2 volumes of 200 µl of pure HPLC grade acetonitrile under positive pressure. Tubes were placed in a 45^{o}C heating block until dry. Acetonitrile was added to the tubes to resuspend the derivatized solid, and filtered into tared 2 ml HPLC sample vials (Agilent), with blue-cap PTFE Rubber septa (Agilent) and acetonitrile was added to 400 mg. Derivatization efficiency was determined by derivatizing known quantities of butyraldehyde and 2-butanone and comparing peak areas generated with the HPLC method described below to the peak areas generated with the same method using Supelco calibration standards.

HPLC analysis of derivatized samples. An Agilent 1200 HPLC with a quaternary solvent pump and a photodiode array detector was used for separating and quantifying the derivatized carbonyl products. The reverse phase separation was done using a Poroshell 120 EC-C18 3 x 75 mm column with 2.7 µm superficially porous particles, and the corresponding 3 x 5mm UHPLC guard column (Agilent) The mobile phase was a 50:45:5 mixture of acetonitrile, water, 5 mM H_2SO_4, flowing at 1 ml min^{-1}. The detector was set to monitor 360 nm absorbance with a bandwidth of 10 and a sampling rate of 40 Hz. Elution times and order were compared to butyraldehyde-2,4-DNPH and butanone-2,4-DNPH calibration standards from Supelco. A calibration curve was generated using known quantities of butyraldehyde and 2-butanone that

were derivatized as described above with acrolein-2,4-DNPH as an internal standard. The ratios of the peak areas each of the derivatized carbonyls to the peak area of the internal standard were plotted against the starting concentrations of the carbonyls in triplicate. Every batch of samples analyzed included 3 control samples in order to verify the calibrated ratios and the derivatization efficiency were behaving consistently.

Supporting info:

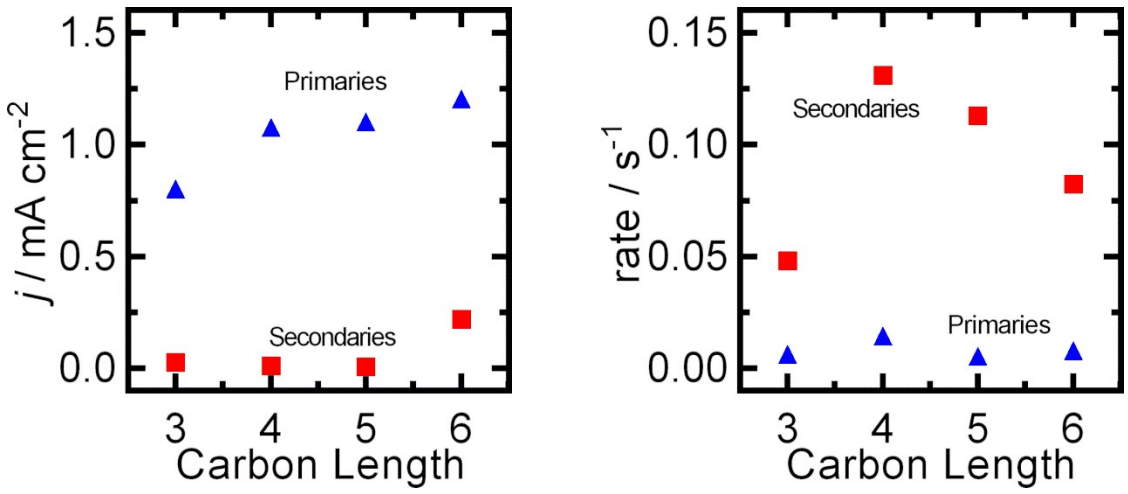

Figure S1. TEMPO and AdhD oxidation of short aliphatic alcohols. 500 mM of 1- and 2-propanol, 1- and 2-butanol, 1- and 2-pentanol, and 1- and 2-hexanol were used as substrates. (Left) Electrochemical currents measured from 1 mM TEMPO-NH_2 in the presence of the primary and secondary alcohols in 137 mM NaCl electrolyte with 10 mM phosphate buffer at pH 8.3. (Right) AdhD oxidation rates of the alcohols with 1 mM NAD^+ in 50 mM glycine buffered to pH 8.8.

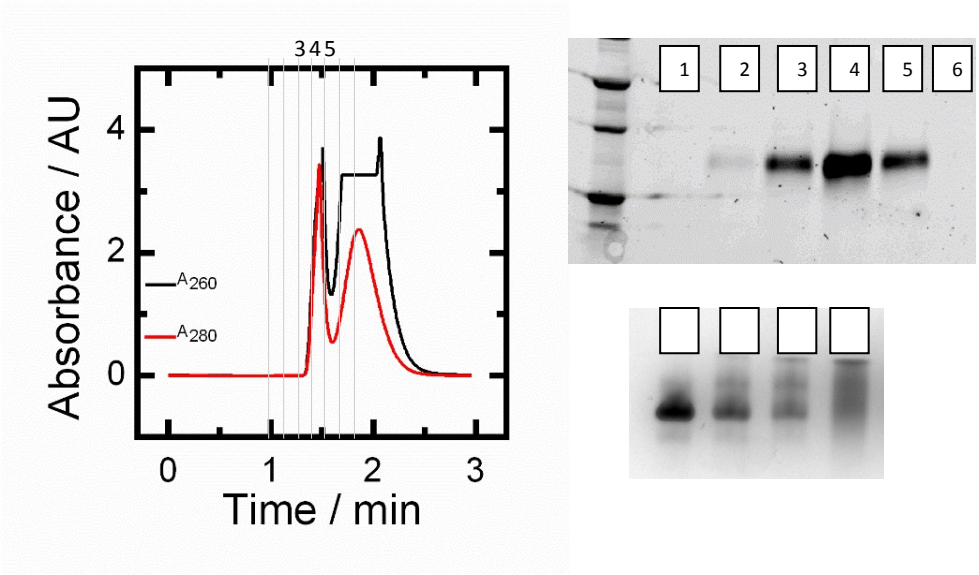

Figure S2. Purification and characterization of AdhD-TEMPO. (Left) Chromatogram showing A260 (black) and A280 (red) from Hi-Trap desalting column purification of AdhD-TEMPO. This purification step was undertaken to remove unreacted 4-glycidyl-TEMPO from AdhD-TEMPO. (Top, right) SDS-PAGE analysis of column purification fractions, gel lanes correspond to the fractions shown in the chromatogram. (Bottom, right) Native-PAGE analysis of AdhD and AdhD-TEMPO. Lane 1 is unmodified AdhD, while lanes 2-4 contain samples of AdhD-TEMPO synthesized with increasing molar excess of 4-glycidyl-TEMPO (100x, 250x, and 500x, respectively).

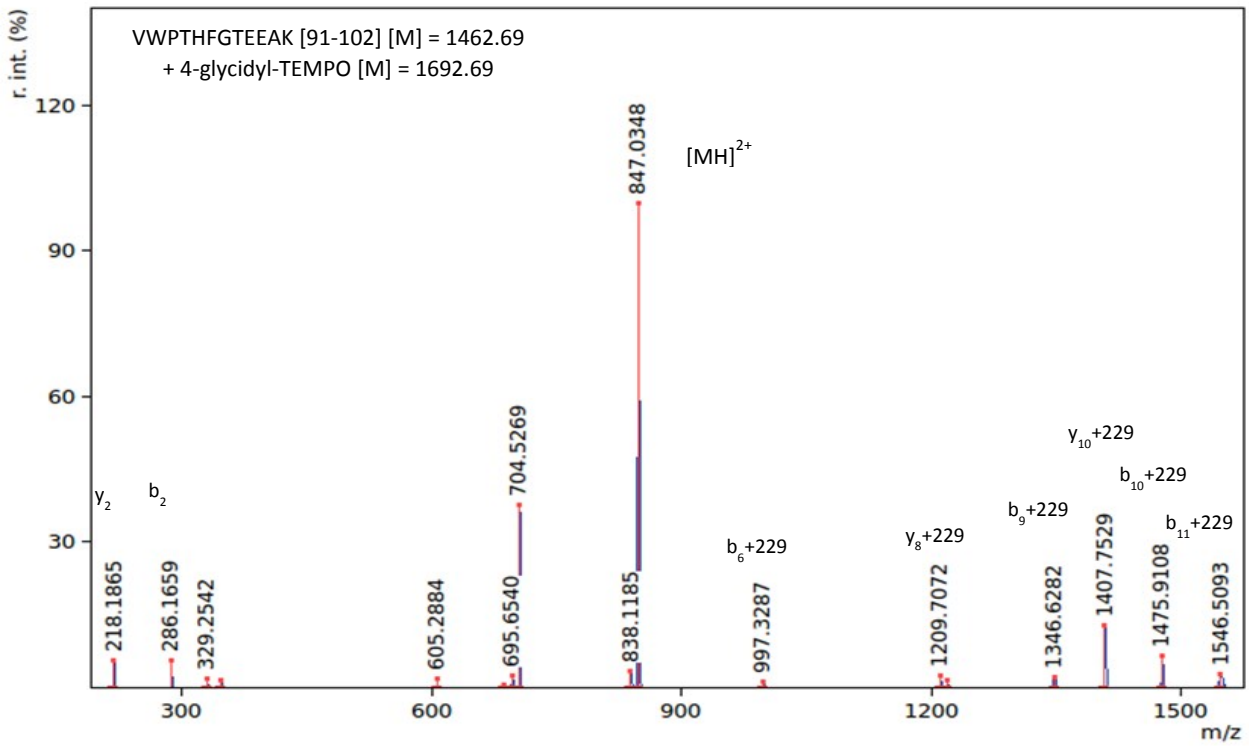

Figure S3. Deconvoluted mass spectra of parent and daughter ions from an AdhD peptide with 4-glycidyl-TEMPO attached at H95. Fragment contains residues 91-102.

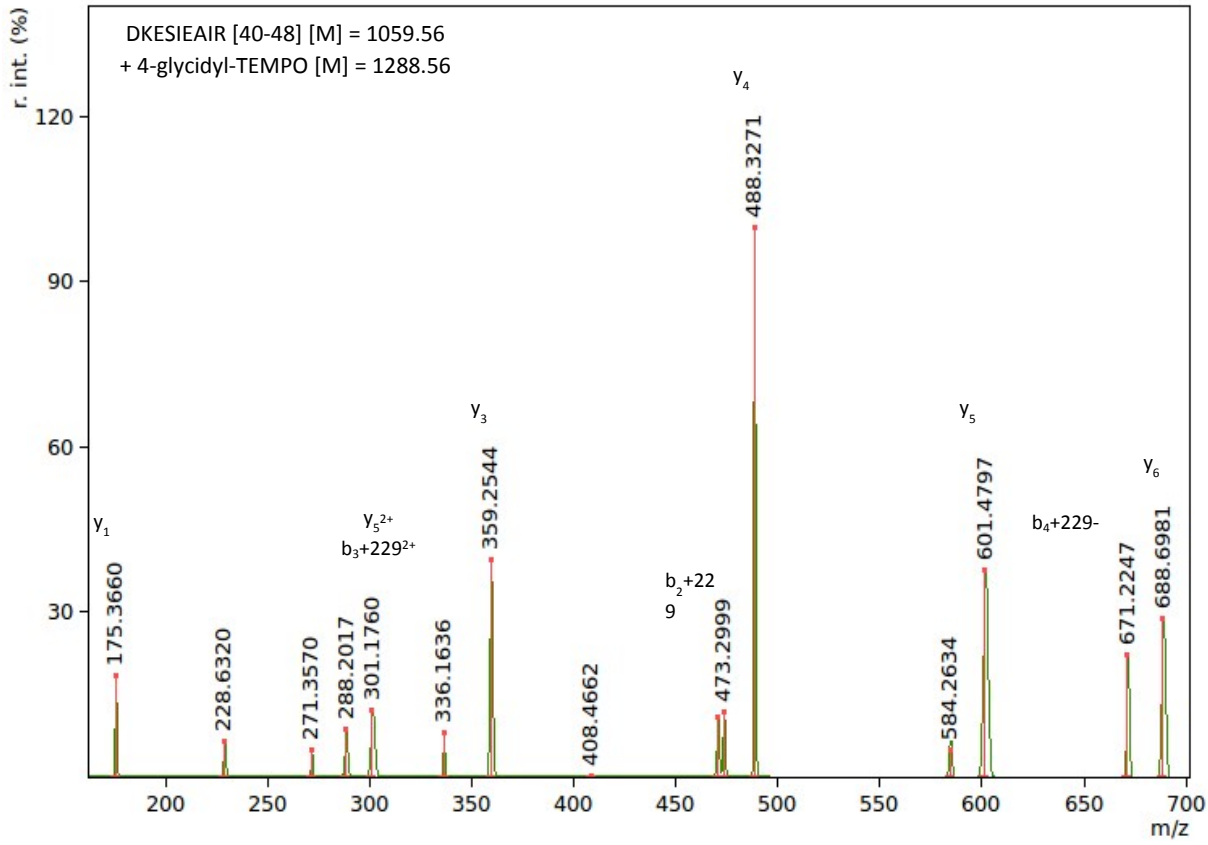

Figure S4. Deconvoluted mass spectra of daughter ions from an AdhD peptide with 4-glycidyl-TEMPO attached at K95. Fragment contains residues 40-48.

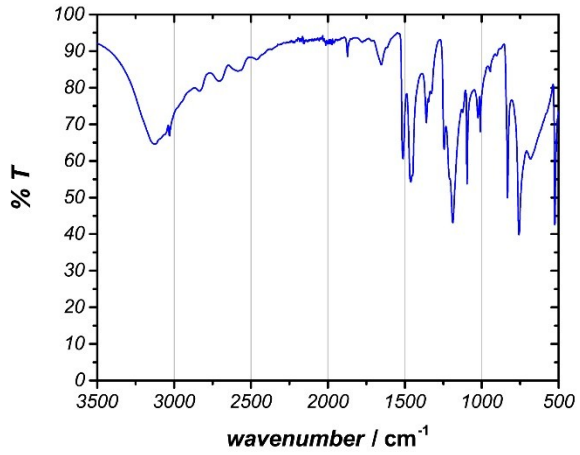

Figure S5. ^{1}H-NMR CD_3OD of DHA-quinoxaline.

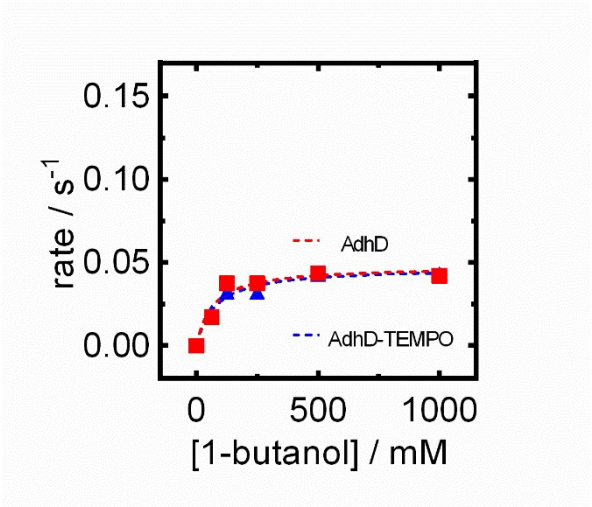

Figure S6. Kinetic analysis of AdhD (red) and AdhD-TEMPO (blue) NAD^+ dependent oxidation of 1-butanol.

Table S1. Kinetic parameters of 1- and 2-butanol oxidation by AdhD and by the AdhD of AdhD-TEMPO

	AdhD		AdhD-TEMPO	
	1-butanol	2-butanol	1-butanol	2-butanol
kcat	0.0477 ± 0.004577	0.1481 ± 0.01029	0.04264 ± 0.006768	0.1883 ± 0.03473
Km	67.61 ± 28.02	202.3 ± 39.99	17.08 ± 26.25	425.5 ± 173.2

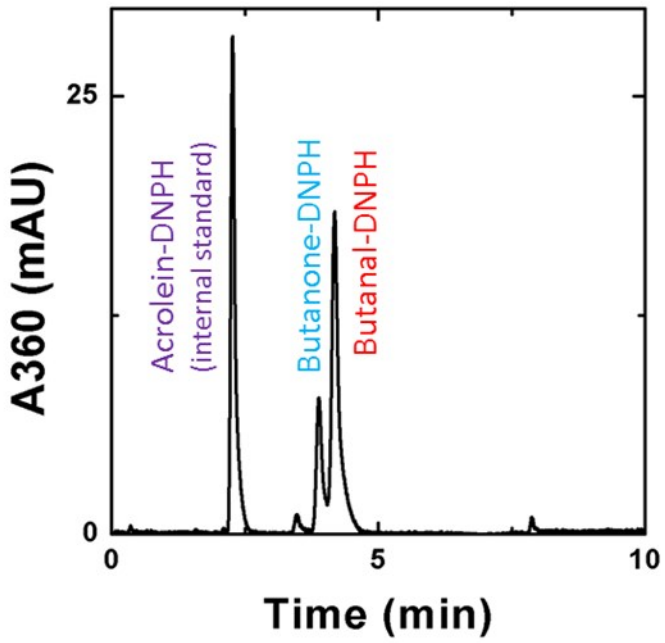

Figure S7. Representative chromatogram of the derivatized reaction mixture when both TEMPO and AdhD catalyze primary and secondary butanol oxidation. The isomer products are separable due to the geometry of the derivatizing agent, 2,4-DNPH, which also allows the product to be measured through the absorbance of 360 nm light.

Table S2. AdhD and TEMPO reaction controls. Conditions of positive and negative control experiments and their relative product yields after 10-minutes of reaction time are indicated. All reactions contain 125 mM each 1-, and 2-butanol.

AdhD	TEMPO	NAD+	800 mV	Butanone	Butanal
+	-	-	-	-	-
+	-	-	+	-	-
+	-	+	-	***	*
+	-	+	+	***	*
-	+	-	-	-	-
-	+	-	+	-	***
-	+	+	-	-	-
-	+	+	+	*	***
+	+	-	-	-	-
+	+	-	+	-	*
+	+	+	-	***	*
+	+	+	+	***	***

+ = component included, - = component not included / no product, * = trace product yield, *** = high product yield

References

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2. C. Song, K. N. Hu, C. G. Joo, T. M. Swager and R. G. Griffin, *J Am Chem Soc*, 2006, **128**, 11385-11390.
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4. U. EPA, *Journal*, 1996.