Electrochemical Recycling of Adenosine Triphosphate in Biocatalytic Reaction Cascades

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Abbreviations

1-$NaSO_3$-AQ - Sodium Anthraquinone-1-sulfonate
2-$NaSO_3$-AQ – Sodium anthraquinone-2-sulfonate
2-$NaSO_3$-3,4-diOH-AQ - 3,4-Dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt
2,6-Bis$NaSO_3$-AQ - disodium 9,10-dioxo-9,10-dihydro-
2,6-anthracenedisulfonate
ADP – Adenosine 5'-diphosphate
AMP – Adenosine 5'-monophosphate
ATP – Adenosine 5'-triphosphate
AcK – Acetate Kinase 101
CK – Creatine Kinase
CV- cyclic voltammetry
DABCO – 1,4-Diazabicyclo[2.2.2]octane
DDQ - 4,5-Dichloro-3,6-dioxo-1,4-cyclohexadiene-1,2-dicarbonitrile
DPPF - 1,1'-Ferrocenediyl-bis(diphenylphosphine)
DtBPF - 1,1'-Bis(di-tert-butylphosphino)ferrocene
FAD – flavin adenine dinucleotide
FcAc – Acetylferrocene
FcAc2 – 1,1'-Diacetylferrocene
FcCOOH – Ferrocenecarboxilic acid
FcCOOH2 – 1,1'-Ferrocenedicarboxylic acid
FcMeOH – Ferrocenemethanol
FcMeOH2 – 1,1'-Ferrocenemethanol
FcNMe2 – (Dimethylaminomethyl)ferrocene
FcNMe3 – *N,N,N*-Trimethyl-1-ferrocenylmethanaminium chloride
GDP – Guanosine 5'-diphosphate
GMP – Guanosine 5'-monophosphate
GTP – Guanosine 5'-triphosphate
HPLC – High-Pressure Liquid Chromatography
Keto-ABNO - 9-Azabicyclo[3,3,1]nonan-3-one-9-oxyl
LCAP - Liquid Chromatography Area Percent
MTR – S-methyl-5-thioribose kinase Rd4BB
Me10-Fc - Decamethylferrocene
PanK – Pantothenate Kinase Rd4BB
PO – Pyruvate Oxidase
PINOH - N-hydroxylphthalimide
PIPES - Piperazine-N,N'-bis(2-ethanesulfonic acid)
TBAOBz - Tetrabutylammonium benzoate
TEMPO - 2,2,6,6-Tetramethyl-1-piperidinyloxy
TEMPO(4Ac) - 4-Acetamido-2,2,6,6-tetramethylpiperidine 1-oxyl
TPP – Thiamine Pyrophosphate
UP – Uridine Phorphorylase Rd6BB
Wt% - weight percent

1. Chemicals and Materials

Unless stated otherwise, all the reagents and solvents were purchased from commercial suppliers (Acros, Merck Millipore Sigma, TCI America, Fisher Scientific, *etc*) and were of the highest analytical purity. *N,N,N*-Trimethyl-1-ferrocenylmethanaminium chloride,[1] 5-isobutylribose **1**,[2] and 2-ethynyl glycerol[3] were synthesized following previously published procedures. D_2O (D, 99.96%) was purchased from Cambridge Isotope Laboratories.

2. Enzyme Preparation

The PO, MTR and UP sequences were already reported, were expressed as previously reported[2] and were used as lysate powders without further purification. The sequence for AcK 101 was reported before[3] and is commercially available from Codexis Inc. and was used as lysate powder without further purification. The sequence for PanK Rd4BB was reported and the enzyme was expressed as previously reported,[3] and was used as lysate powder without further purification. CK from rabbit muscle was purchased from Sigma Aldrich, Catalase from *Corynebacterium glutamicum* was purchased from Roche. YwfE was produced according to the following procedure: the gene encoding for YwfE bearing a C-terminal His-tag was introduced into pET24a(+) in E. coli BL21(DE3). Cells were grown in 1 L TB medium, and protein was expressed at 18 C for 18h following induction with 0.5 mM IPTG. Cells were lysed in 25 mM Tris-HCl pH 8.0 using a microfluidizer, and the lysate was clarified and lyophilized to give a cell-free powder.

3. Enzyme Purification

PO was purified on HisPur™ Ni-NTA Spin Columns (Thermo Fisher Scientific, part number 88226) using the manufacturer's procedure. Purified protein was buffer exchanged to potassium phosphate buffer (50 mM, pH 7.5) using Amicon Ultra-15 Centrifugal Filter Unit (30 KDa, Millipore, part number UFC903008). The protein concentration was calculated based on its absorbance at 280 nm (A280) and the calculated extinction coefficient for the protein (78395 $cm^{-1}.M^{-1}$ from ExPASy).

4. Analytical Instrumentation

^1H NMR spectra were recorded on a 500 MHz Bruker UltraShield spectrometer and chemical shifts are reported in ppm and referenced to the HOD peak at 4.79 ppm. HPLC was performed using Waters Acquity UPC2 instrument equipped with a UV detector or a Waters Acquity instrument equipped with a UV detector.

5. Electrolysis Equipment

Small scale (<0.5 mL) electrochemical experiments were conducted using the HT*e*-Chem[4] set up equipped with graphite anodes and stainless steel cathodes. Mid-small scale (<5 mL) synthetic electrochemical experiments were conducted using an IKA Electrasyn 2.0 (part number 0020008980) using 5 mL vials/caps (part number 0040003171). For multiple parallel reaction, the Electrasyn was outfitted with an IKA carousel (part number 0020017512). Graphite electrodes (part number 00400002858) were used as anodes and stainless steel (part number 00400002851) were used as cathodes in an undivided cell. The graphite electrodes were prepared by first soaking in a ~0.5 M KOH solution, then washing abundantly with water and polishing with a wet 80-grit sandpaper, followed by sonication in water, then acetone and dried under vacuum overnight. Using this cleaning procedure, the electrodes were then reused for many reactions. The stainless steel electrodes were washed abundantly with water and polished with a wet 80-grit sand paper, followed by sonication in water, then acetone and air dried.

Larger scale batch (80 mL) synthetic electrochemical experiments were conducted using a Mettler Toledo EasyMax 102 (part number 51161711) with a two-piece 100 mL EasyMax vessel (part number 51161708). A vessel cover featuring 8 0.25" holes and 2 probe holes was 3D-printed to be fitted on top of the 100 mL vessel while allowing electrodes to dip in the solution. The electrode set up consisted of 4 graphite electrodes (0.24"x6", McMaster-Carr, part number: 9121K71, polished with an 80-grit sandpaper), 3 stainless steel electrodes (0.24", McMaster-Carr, part number: 89535K85) and a BASi RE-5B Ag/AgCl (3M NaCl) Reference Electrode (Part number:MF-2052) that were placed in the 0.25" holes in the 3D-printed vessel cover. The calculated total electrode surface area from CV of a 1 mM FcCOOH in 0.1 M KPi buffer at ph 7 was determined to be 27 cm^2. The distance between the electrodes was designed

to be 4 mm like the electrode distance in the Electrasyn. The electrodes were connected to an EmStat 4S HR from BASi (West Lafayette, Indiana) and the data was recorded using PSTrace (version 5.9.1808) software.

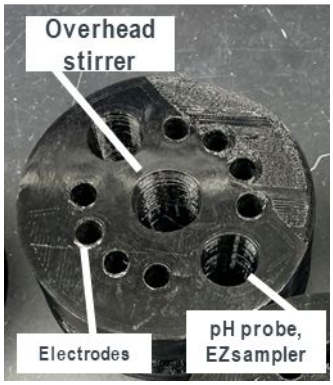


Figure S1: Picture of 3D-printed EasyMax vessel cover. The smaller holes are 0.25" to fit the electrodes and the larger holes fit various probes. The central hole fits the overhead stirrer.

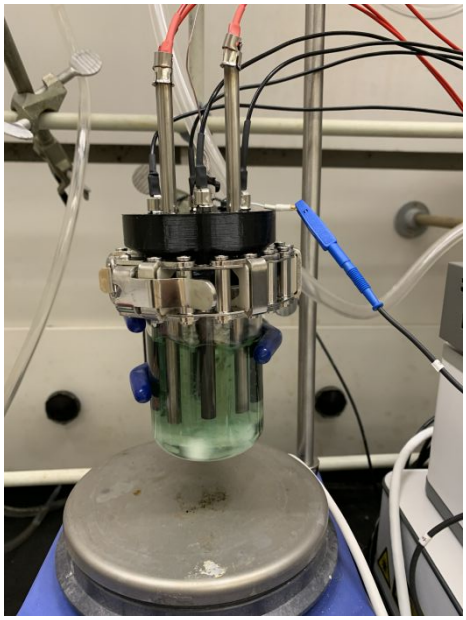


Figure S2: Side view of 120 mL EasyMax vessel outfitted with the vessel cover equipped with the graphite and stainless steel electrodes. Note: for the bulk electrolysis reactions, the electrodes were pushed down to the bottom of the vessel.

The large-scale flow electrochemical experiment was conducted in an Electrocell MP cell equipped with a resin-filled graphite plate diamond polished to 3000 grit as the anode and a stainless steel cathode. The electrodes were spaced to 4 mm using a custom made Teflon gasket and static mixer. The calculated electrode surface area from CV of a 1 mM FcCOOH in 0.1 M KPi buffer at ph 7 is 256 cm^2. A BK Precision 9206 Multi-Range DC Power Supply provided the current and a Quattroflow QF1200 4-diaphragm pump was used to circulate the liquid in a recirculation loop going in and out of a Mettler Toledo Optimax 1001 (part number 51162701).

6. HPLC analytical method

6.1.Method A: HPLC Method for ATP and GTP conversion:

- Mobile phase A: 0.1 M triethylammonium in water, pH 7, Mobile phase B: Acetonitrile, Flow rate: 0.6 mL/min, temperature: 30 °C, Absorbance: 260 nm, Column: HSS T3 Column, 100Å, 1.8 µm, 2.1 mm X 50 mm (part number: 186003538), method run time: 4 min
- Gradient:

time (min)	%A	%B
0	99	1
3	92	8
3.1	99	1

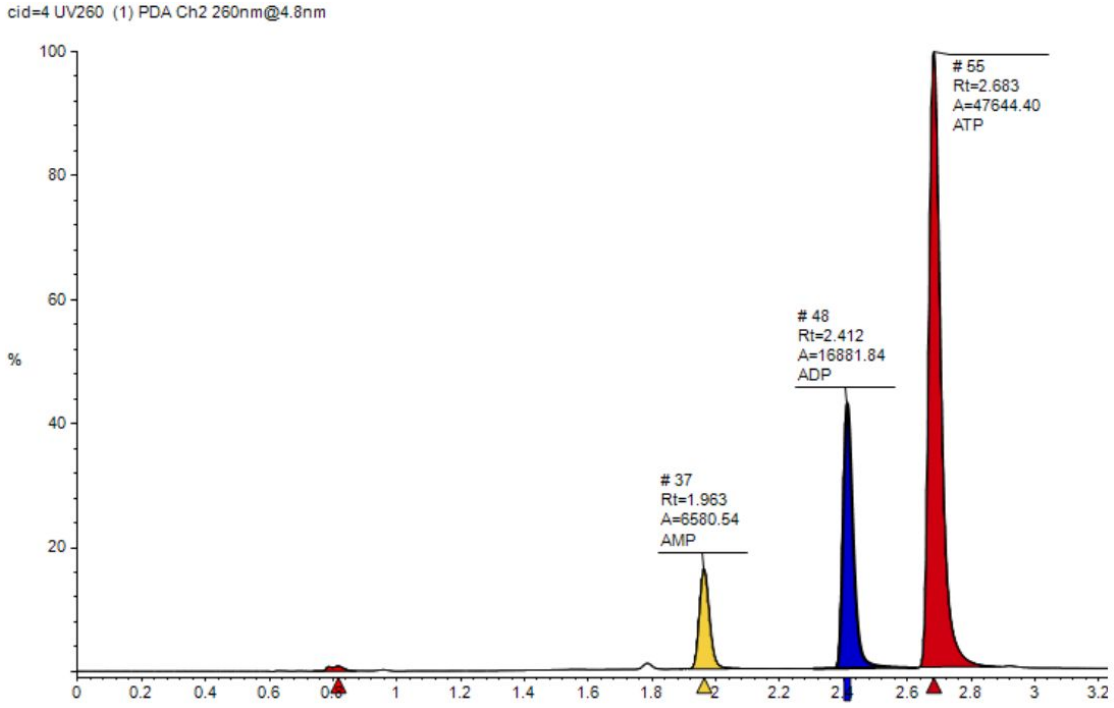


Figure S3: Chromatogram of a mixture of AMP, ADP and ATP separated using method A.

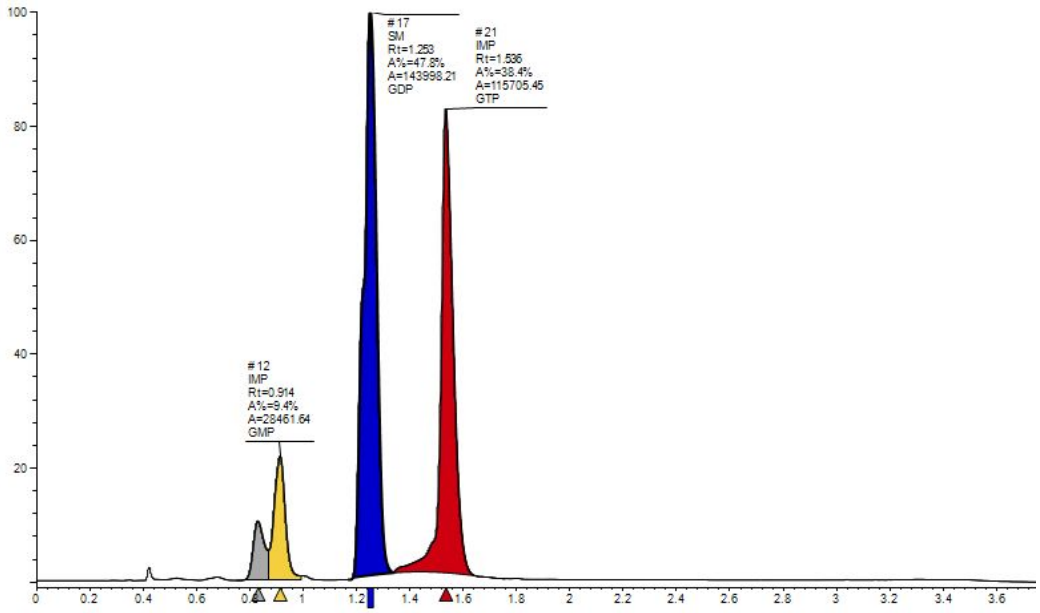


Figure S4: Chromatogram of a mixture of GMP, GDP and GTP separated using method A.

6.2. Method B: HPLC Method for molnupiravir step 2 Uracil (glycosilation) conversion:

- Mobile phase A: water, 0.1 % trifluoroacetic acid, Mobile phase B: Acetonitrile, Flow rate: 1.25 mL/min, temperature: 25 °C, Absorbance: 270 nm, Column: XBridge Glycan BEH Amide Column, 130Å, 3.5 µm, 4.6 mm X 150 mm (part number: 186007275), method run time: 9 min total with post run
- Gradient:

time (min)	%A	%B
0	5	95
3	16.4	83.6
4	80	20
6	80	20
6.1	5	95

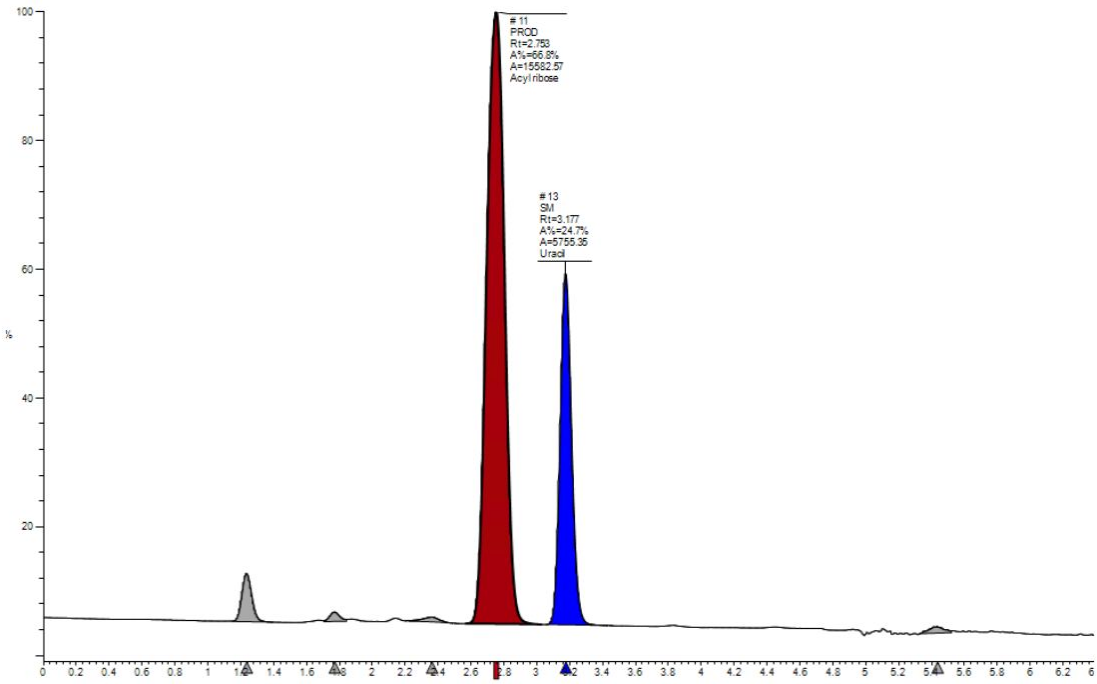


Figure S5: Chromatogram of a mixture of acyl-ribose **3** and uracil separated using method B.

6.3. Method C: LCMS method for Phe-Ser coupling

- Mobile phase A: water, 2 mM ammonium formate, 1.9 mM formic acid, Mobile phase B: 90:10 Acetonitrile:water, 2 mM ammonium formate, 1.9 mM formic acid, Flow rate: 0.8 mL/min, temperature: 50 °C, Absorbance: 210 nm, Column Acquity UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 50 mm, (part number: 186002350), method run time: 6 min
- Gradient:

time (min)	%A	%B
0	95	5
5.5	1	99
5.9	1	99
5.91	95	5

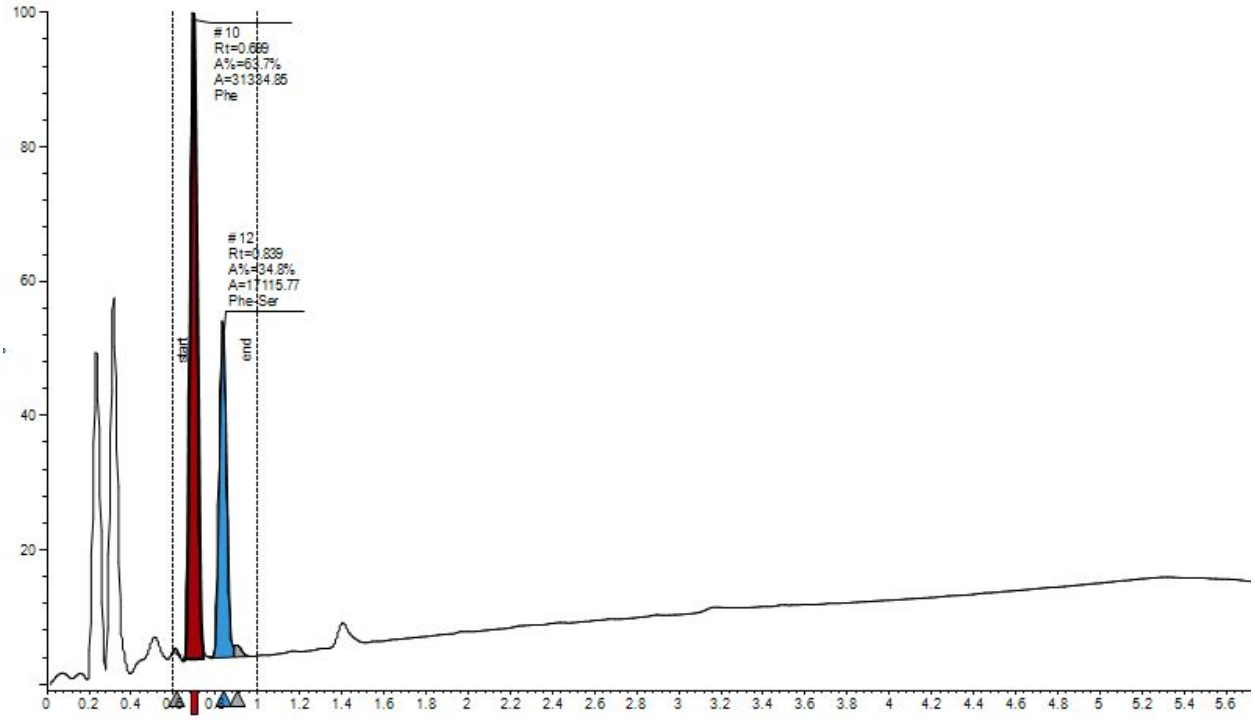


Figure S6: Chromatogram of a mixture of Phe and Phe-Ser separated using method C. The mass of each component was confirmed by MS: m/z for [Phe -H]: 164.1, [Phe-Ser -H]: 251.2.

7. Determination of Conversion and Faradaic Efficiency

7.1. ATP conversion: ATP, ADP and AMP were the only peaks integrated in method A. Since ATP, ADP and AMP have the same extinction coefficient ad 260 nm (15,400 cm-1.mol-1, obtained from Sigma Aldrich and Oriental yeast Co., Ltd.), we can use the formula:

$$conversion\ to\ ATP = \frac{LCAP_{ATP}}{LCAP_{ATP} + LCAP_{ADP} + LCAP_{AMP}}$$

The same method was used to calculation GTP conversion

7.2. Uracil Conversion: Uracil and **3** were both the only peaks integrated in method B. It should be noted that uracil and **3** have different extinction coefficients at 270 nm, and therefore, the LCAP need to be normalized to calculate the conversion

$n_{uracil} = LCAP_{uracil} \times 1.29$

$n_{\mathbf{3}} = LCAP_{\mathbf{3}} \times 0.71$

$$uracil\ conversion = \frac{n_{\mathbf{3}}}{(n_{\mathbf{3}} + n_{uracil})} \times 100$$

7.3. Phe-Ser conversion:

$$Phe - Ser\ conversion = \frac{LCAP_{Phe-Ser}}{(LCAP_{Phe-Ser} + LCAP_{Phe})} \times 100$$

7.4. Faradaic efficiency: $Faradaic\ efficiency = \frac{charge\ converted\ to\ desired\ product}{total\ charge\ passed}$

In this study:

$$Faradaic\ efficiency = \frac{\frac{conversion\ in\ \%}{100} 2nF}{It}$$

Where n is the number of moles of starting material, F is the Faraday constant, I is the current in A and t is the time elapsed in s. The factor of 2 is added to account for the fact that 2 electrons are required to produce 1 molecule of ATP

8. UV-Vis Determination of FAD:PO ratio

The UV-Vis system used to record spectra consists in an OceanInsight DH-2000-BAL light source directly connected to an OceanInsight SQUARE ONE cuvette holder using threaded joints. The cuvette holder was directly connected to an OceanInsight FLAME-S-UV-VIS spectrometer. The spectra were recorded using OceanView 2. The exposure time was automatically adjusted to be under the saturation limit of the spectrometer. 10 scans were averaged to produce a spectrum. A background of the light source and a dark were taken before each experiment.

In a 1.4 mL Thorlabs quartz cuvette (CV10Q14), dilute 50 µL of purified pyruvate oxidase stock solution with 950 µL of water and take an UV-vis spectrum. Using the known absorption of FAD at 450 (ε = 11,300 $cm^{-1}.mol^{-1}$),[5] the FAD concentration was determined and the resulting absorption from FAD at 280 nm (ε = 22148 $cm^{-1}.mol^{-1}$) was calculated. The result was subtracted from the total absorption at 280nm of PO solution and the enzyme concentration was determined using the calculated extinction coefficient for PO at 280 nm (ε = 78395 $cm^{-1}.mol^{-1}$) and was multiplied by 20 to get to the stock solution concentration. The initial purified PO solution was determined to be 500 µM in PO and 381 µM in FAD (0.76 FAD:PO), and therefore the PO is unsaturated in FAD. 20 µL increments of 1 mM FAD stock were added to the PO stock until the concentration measured by the method described above shows a concentration of FAD higher that of PO. The reached final PO concentration was 461 µM and final FAD concentration was 494 µM.

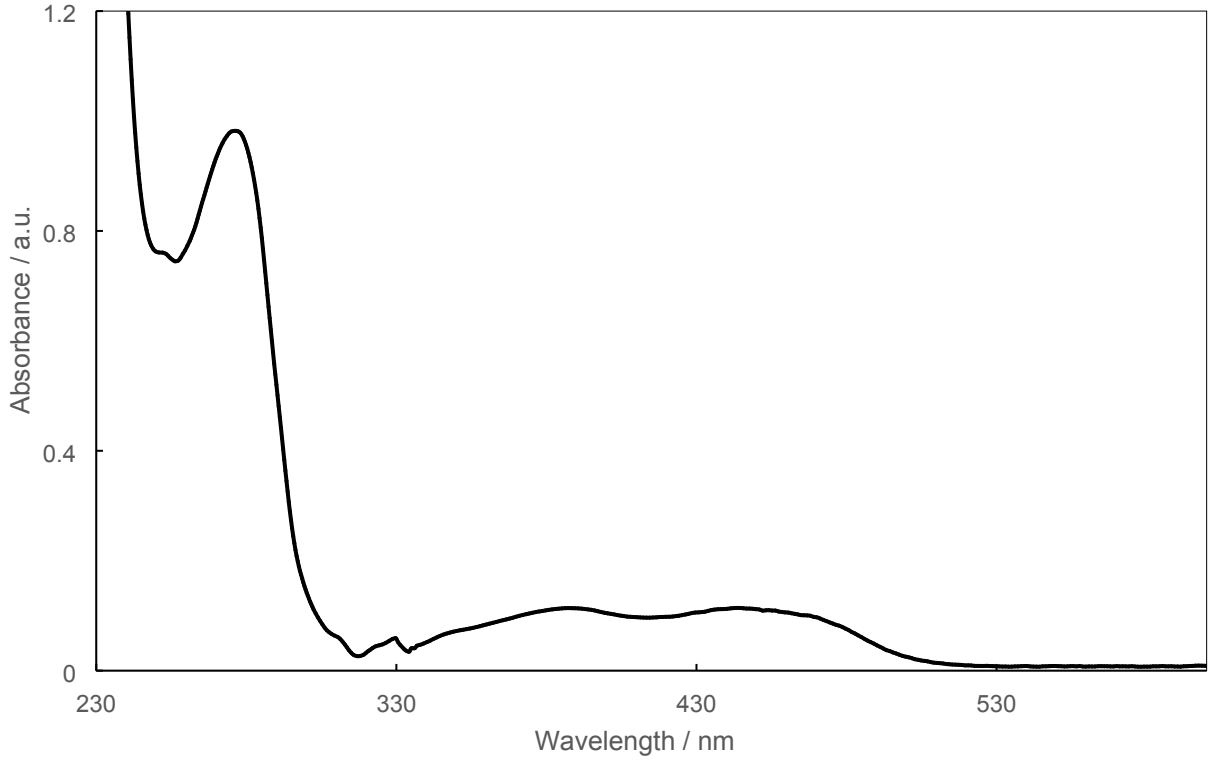


Figure S7: UV-vis spectrum of 20x dilution of purified PO stock solution. FAD concentration (0.49 mM) and PO concentration (0.46 mM). Note: the peak at 330 nm is an instrument artifact.

9. Electrochemical Studies.

Electrochemical measurements were performed with a MultiPalmSens4 PALM-MPS4.F0.05 from BASi (West Lafayette, Indiana) using MultiTrace 4 (version 4.2.2312) software. Cyclic voltammetry (CV) experiments were conducted under N_2, *i.e.* the electrochemical cells were purged for 2 min by vigorously bubbling N_2 in the solution, then maintaining a constant purge of N_2 during the CV measurements. The three-electrode system consisted of a glassy carbon button working electrode (area = 0.071 cm^2) from BASi (Part #: MF-2012), a BASi RE-5B Ag/AgCl (3M NaCl) Reference Electrode (Part #:MF-2052), and a Pt wire counter electrode.

9.1.Sample preparation

The CV samples were prepared as following:

a) First, a pyruvate stock solution was prepared as such: 5 mmol of sodium pyruvate (550 mg) was dissolved in 9 mL of H2O. 5 mmol of potassium phosphate monobasic (680 mg), and 0.1 mL of 1M $MgCl_2$. 8 M KOH was added dropwise under stirring until the pH reaches 6. Then, water was added until the solution is 10mL in total volume. 4.3 mg of TPP was added (0.01 mmol). Final concentrations: 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, and 1 mM TPP

b) A 10 mM stock solution of mediator was prepared by dissolving 0.01 mmol of mediator (2.16 mg for FcMeOH, or 2.5 mg FcMeOH2, 2.3 mg FcAc, 2.7 mg FcAc2, 2.3 mg FcCOOH, 2.7 mg FcCOOH2, 2.4 mg FcNMe2, 2.9 mg FcNMe3) in 1 mL of EtOH.

b) 1.94 mL of pyruvate stock was placed in a electrochemical cell (VC-2 from BASi), and 40 µL of 10 mM mediator was added. The CV was recorded for the mediator alone, at various scan rates. After that, 20 µL of 461 µM purified PO5 stock was added (ratio FAD:PO 1:1).

A similar procedure was used for the pH dependence experiment, with the only difference being the use of either pH 6, 6.5, 7, and 7.5 and FcMeOH as the mediator.

A similar procedure was used for the experiment with PO unsaturated in FAD, with the only difference being the use of the initial purified PO stock in which the ratio FAD:PO was measured to be 0.71:1 FAD:PO.

Note: The purified PO has limited stability overtime, even when stored at -20°C, and seems to also loose activity when submitted to freeze/thaw cycles. Over ~1 year storage, the activity lost was ~43%. When we repeated CV experiments with older purified PO, we run a study with FcMeOH and using the rate constant for FcMeOH we can simulate the concentration of active enzyme. We then used this new concentration for the data analysis.

9.2. Data collection and analysis

All potentials are reported vs Ag/Ag^+ (in 3M NaCl). The scans were started at either 0 V or 0.2 V and the scan was run towards higher positive potential (towards oxidation) and the potential window was adjusted based on the mediator potential. The scan rate was varied between 5, 10, 20, 50, 100 and 200 $mV.s^{-1}$. The peak or catalytic current was determined using MultiTrace peak finder algorithm. The rate of electron transfer between the enzyme and the mediator were calculated using the following equation.

$$\frac{i_p}{i_p^0} \approx \frac{\sqrt{\lambda}}{0.446}\sqrt{\frac{2}{\sigma}\left[1-\frac{1}{\sigma}ln\,(1+\sigma)\right]} \quad (S3)$$

With i_p being the catalytic current, i_p^0 the peak current without enzyme,

$$\lambda = \frac{2k_c C_E^0 RT}{Fv} \quad (s4)$$

Where k_c is the rate between the mediator and the enzyme, C_E^0 is the enzyme concentration, R is the gas constant, T is the temperature, F is Faraday's constant and v is the scan rate,

$$\sigma = \frac{k_c C_P^0}{k_2}\left(1 + \frac{k_{-1} + k_2}{k_1 C_S^0}\right) \quad \text{(S5)}$$

Where C_P^0 is the mediator concentration, C_S^0 is the substrate concentration, k_1, k_{-1}, k_2 are the rates for the following reactions

$$E + S \overset{k_1}{\rightarrow} ES$$

$$ES \overset{k_{-1}}{\longrightarrow} E + S$$

$$ES \overset{k_2}{\rightarrow} P$$

When $\sigma \rightarrow 0$, the equation simplifies to

$$\frac{i_p}{i_p^0} \approx \frac{\sqrt{\lambda}}{0.446} \quad \text{(S6)}$$

Plotting $\frac{i_p}{i_p^0}$ vs $\sqrt{\frac{2C_E^0 RT}{Fv}}$ gives a linear plot from which k_c is the slope and which was extracted by linear fitting with the intersect set to 0.

The redox potential of each mediator was calculated by averaging the potential of the oxidation peak and the reduction peak in the reversible CV trace:

$$E_{mediator}^0 = \frac{E_{ox} + E_{red}}{2} \quad \text{(S7)}$$

For synthetic scale experiments, the surface area of graphite was determined using cyclic voltammetry by measuring the peak height of a 6.25 mM of FcNMe3 and using the equation:

$$i_{Fc}^0 = 0.446 FSC_{Fc}^0 \sqrt{D} \sqrt{\frac{Fv}{RT}} \quad \text{(S1)}$$

where i_{Fc}^0 represent the peak current of FcNMe3, F the Faraday constant, S the electrode surface, C_{Fc}^0 the ferrocene concentration, D the diffusion coefficient of ferrocene (for $FcNMe_3$ 3.7 x 10^{-6} $cm^2.s^{-1}$),[1] v the scan rate, R the gas constant, and T the temperature.

The same diffusion coefficient was used for all mediators

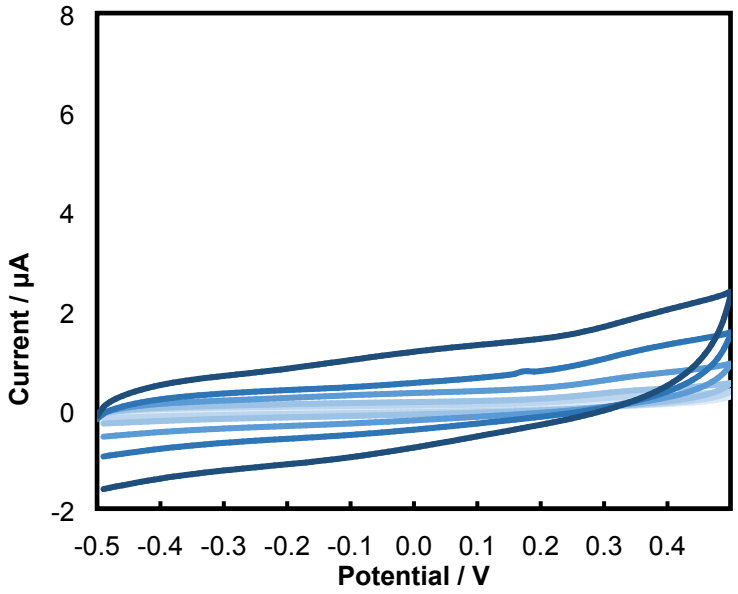


Figure S8: CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

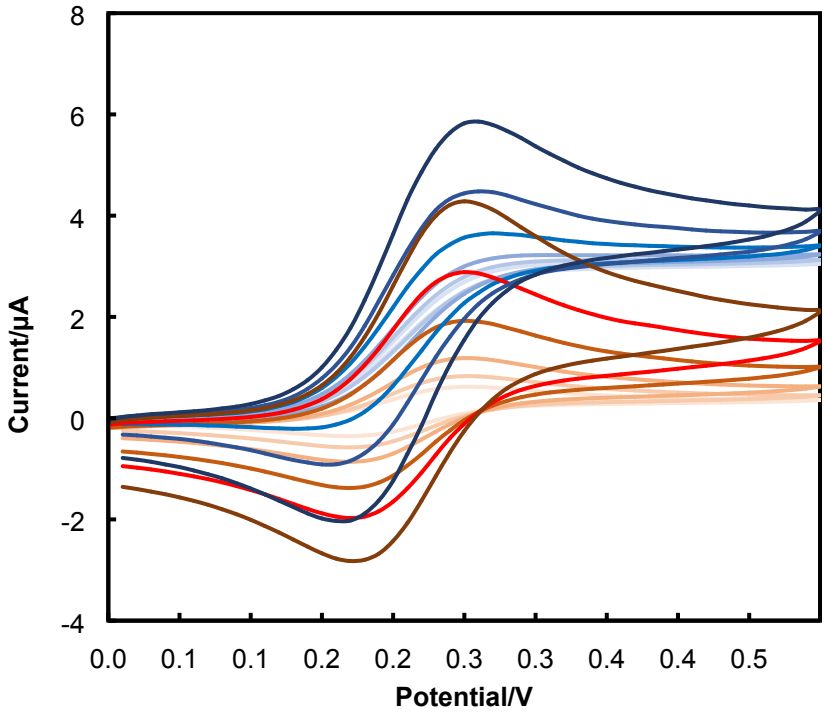


Figure S9: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcMeOH: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcMeOH and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

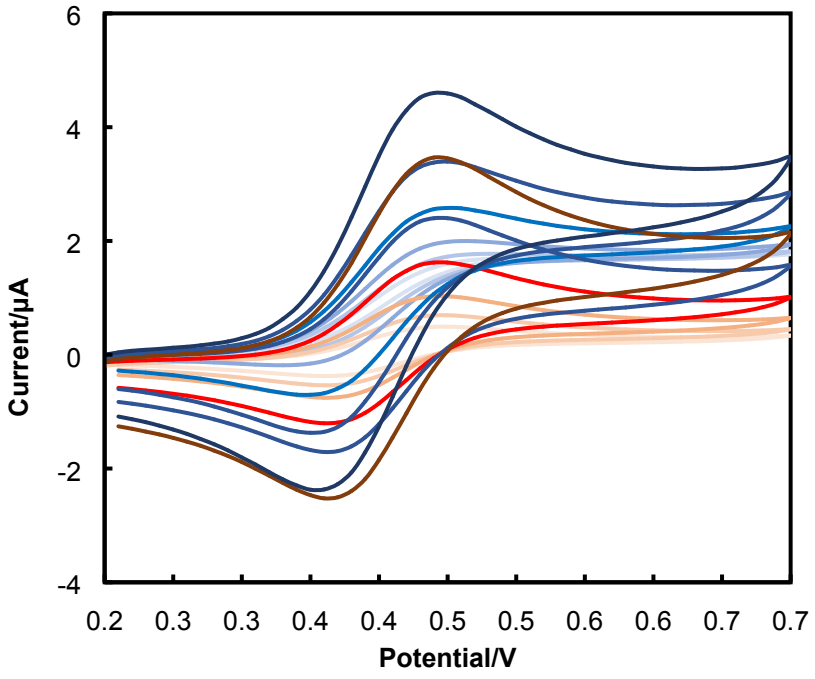


Figure S10: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcNMe3: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcNMe3 and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

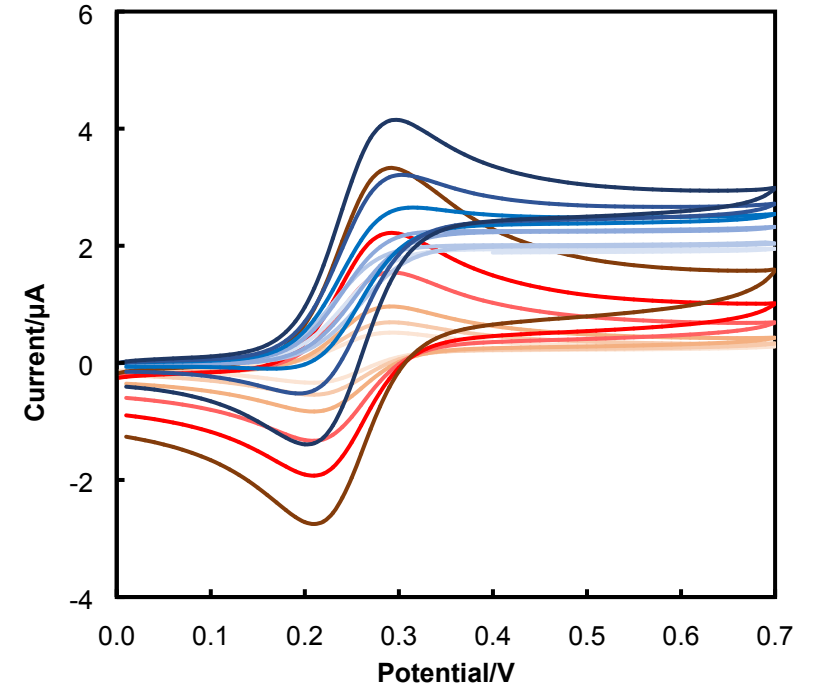


Figure S11: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcMeOH2: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcMeOH2 and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

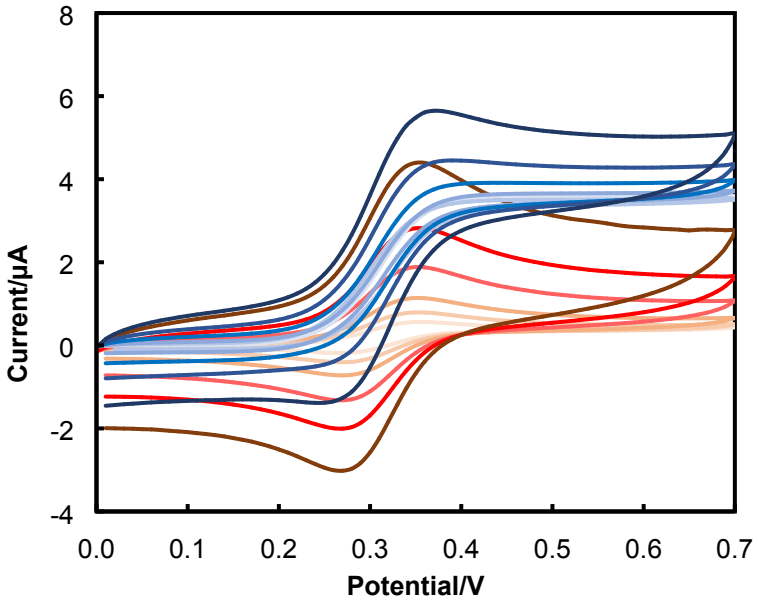


Figure S12: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcCOOH: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcCOOH and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

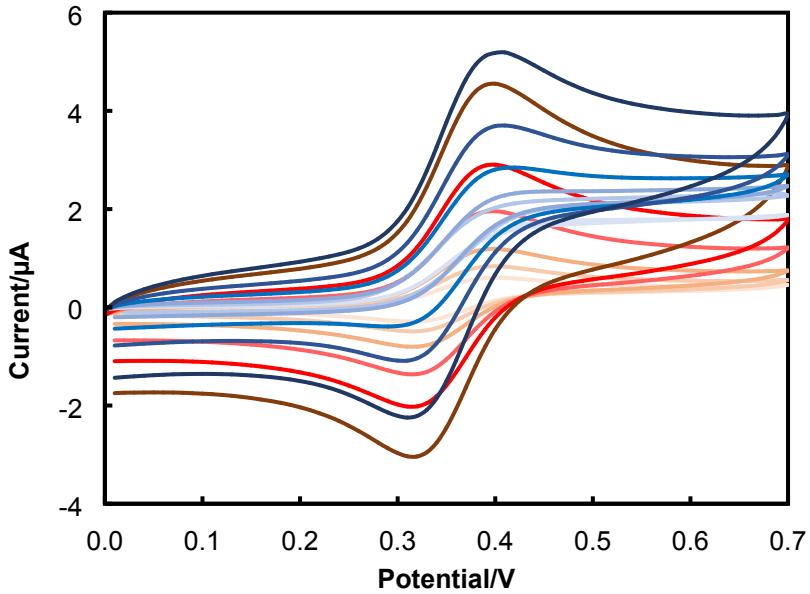


Figure S13: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcNMe2: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcNMe2 and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

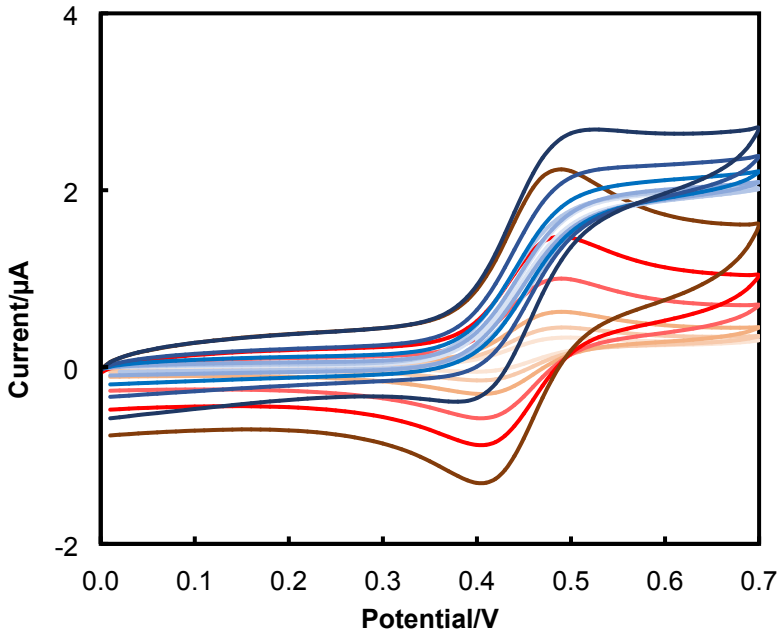


Figure S14: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcCOOH2: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcCOOH2 and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

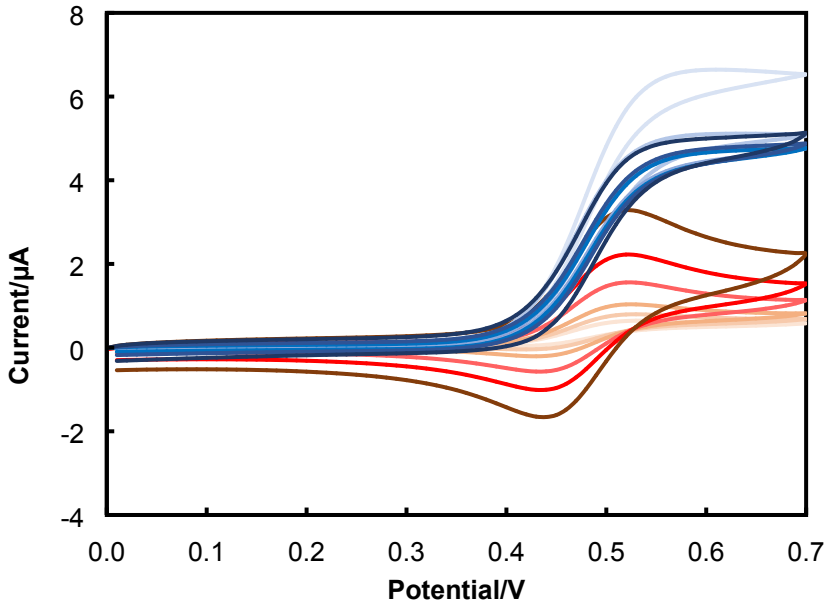


Figure S15: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcAc: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcAc and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$.

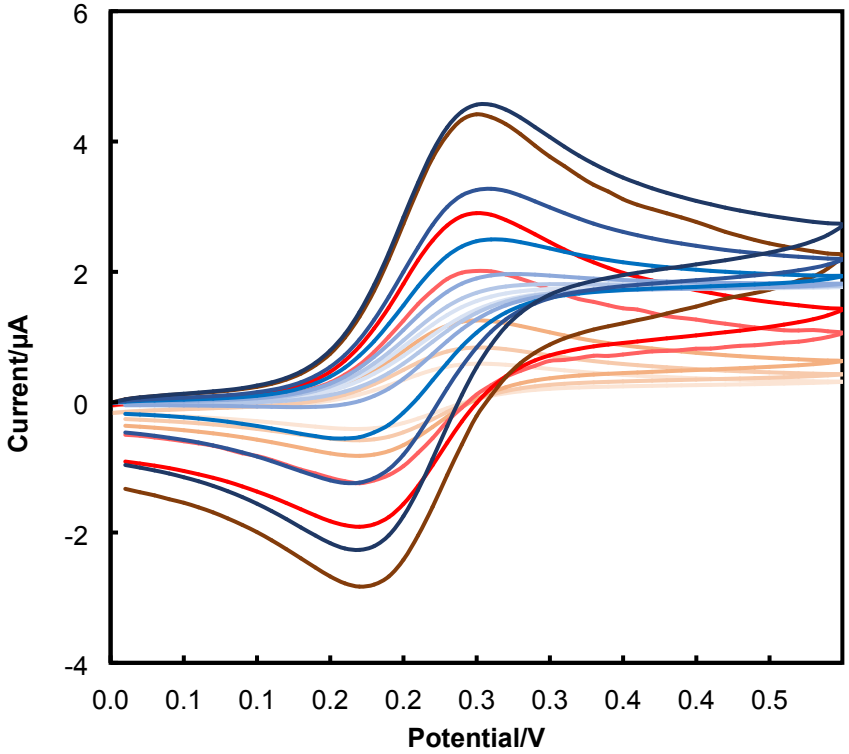


Figure S16: CV of 0.5M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcMeOH: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. CV of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, 0.2 mM FcMeOH and 5 µM PO. Scan rate: (—) 5 $mV.s^{-1}$, (—) 10 $mV.s^{-1}$, (—) 20 $mV.s^{-1}$, (—) 50 $mV.s^{-1}$, (—) 100 $mV.s^{-1}$, (—) 200 $mV.s^{-1}$. The PO used in this experiment has a 0.76:1 FAD:PO ratio.

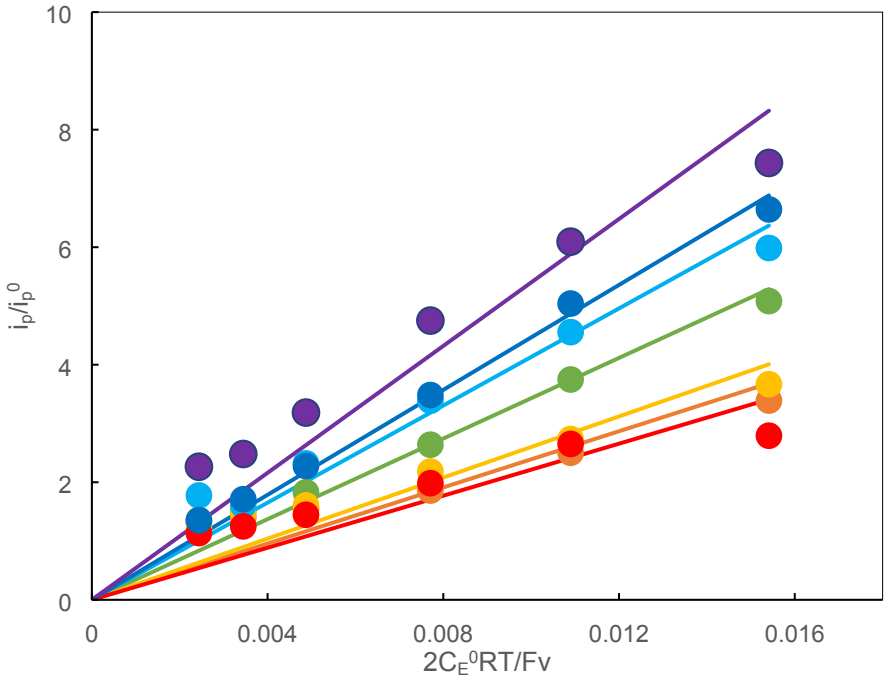


Figure S17: Plot of $\frac{i_p}{i_p^0}$ vs $\frac{2C_E^0RT}{Fv}$ for one run of FcAc (•) and linear fit (—); FcCOOH (•) and linear fit (—); FcCOOH2 (•) and linear fit (—); FcMeOH (•) and linear fit (—), FcMeOH2 (•) and linear fit (—), FcNMe3 (•) and linear fit (—), FcNMe2 (•) and linear fit (—).

Table S1: Redox potential and rate constants for the different mediators.

Mediator	***E⁰ (V vs Ag/AgCl)***	***k ($M^{-1}.s^{-1}$)***	***log(k)***
FcMeOH	0.22	1.02 (±0.1) × 10^5	5.01
FcMeOH2	0.31	1.16 (±0.3) × 10^5	5.24
FcCOOH	0.22	1.52 (±0.1) × 10^5	5.07
FcNMe2	0.36	2.21 (±0.3) × 10^4	4.23
FcNMe3	0.43	7.4 (±0.3) × 10^4	4.74
FcCOOH2	0.45	1.79 (±0.1) × 10^5	5.23
FcAc	0.48	9.00 (±3.4) × 10^5	5.20
FcMeOH (0.76:1 FAD:PO)	0.22	4.16 (±0.4) × 10^4	4.61

Table S2: rate constants for FcMeOH at various pH.

pH	***k ($M^{-1}.s^{-1}$)***	***log(k)***
6	1.02 (±0.1) × 10^5	5.01
6.5	1.19 (±0.1) × 10^5	5.07

7	$1.64\ (\pm 0.2) \times 10^5$	5.21
7.5	$1.7\ (\pm 0.2) \times 10^5$	5.23

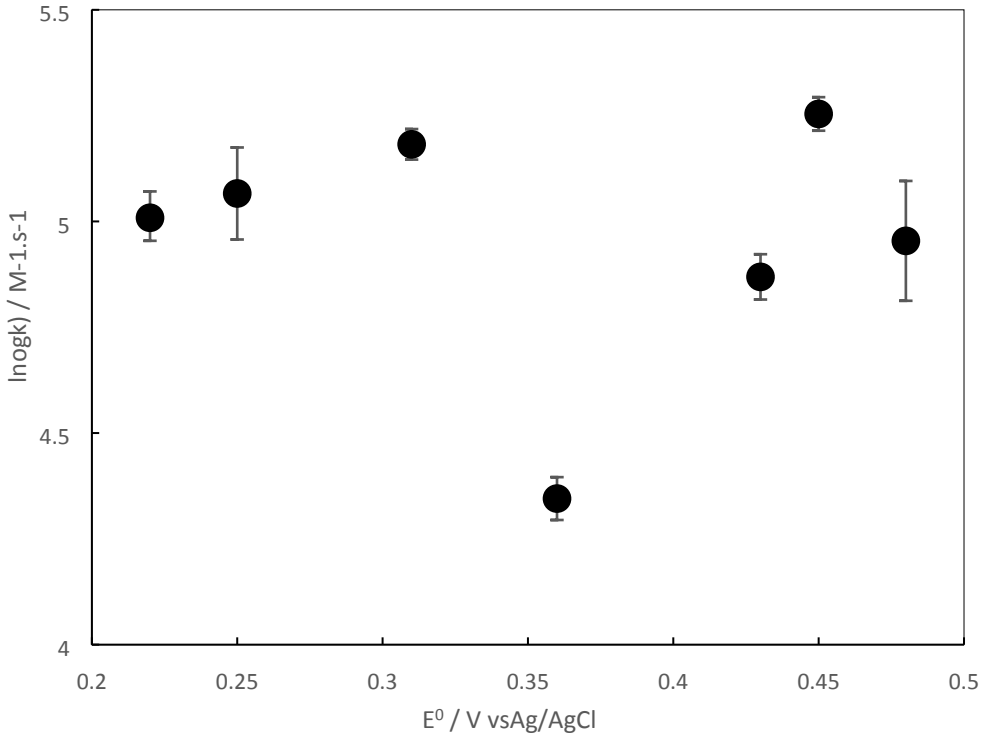


Figure S18: Plot of log(k) against $E^o_{mediator}$ (•).

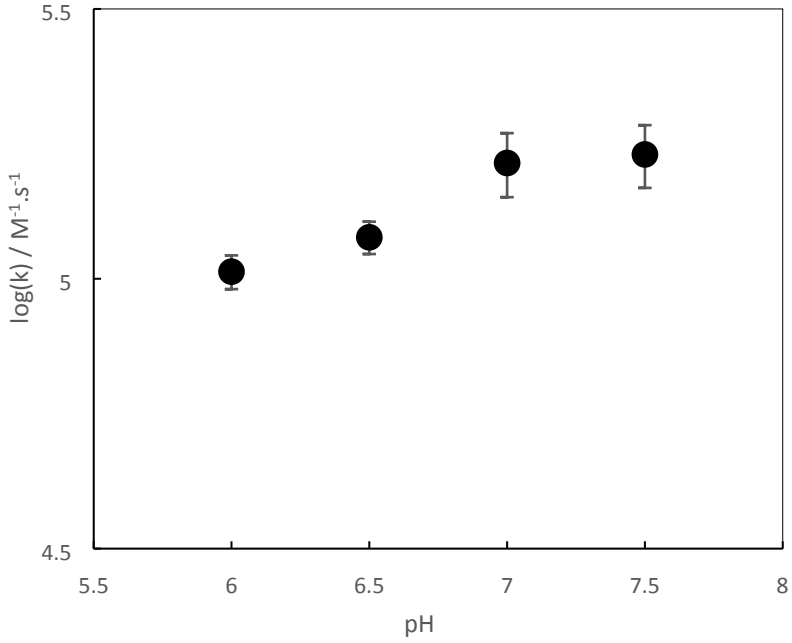
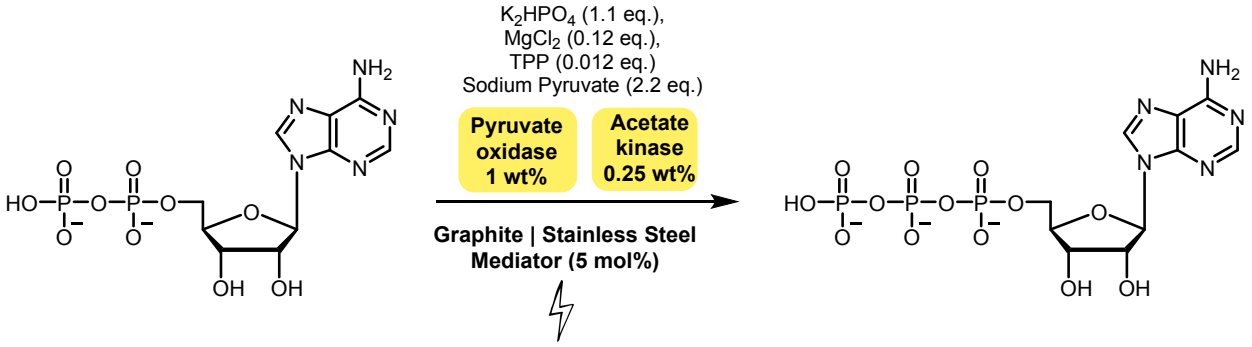


Figure S19: Plot of log(k) against pH (•).

10. Bioelectrochemical stoichiometric ATP formation

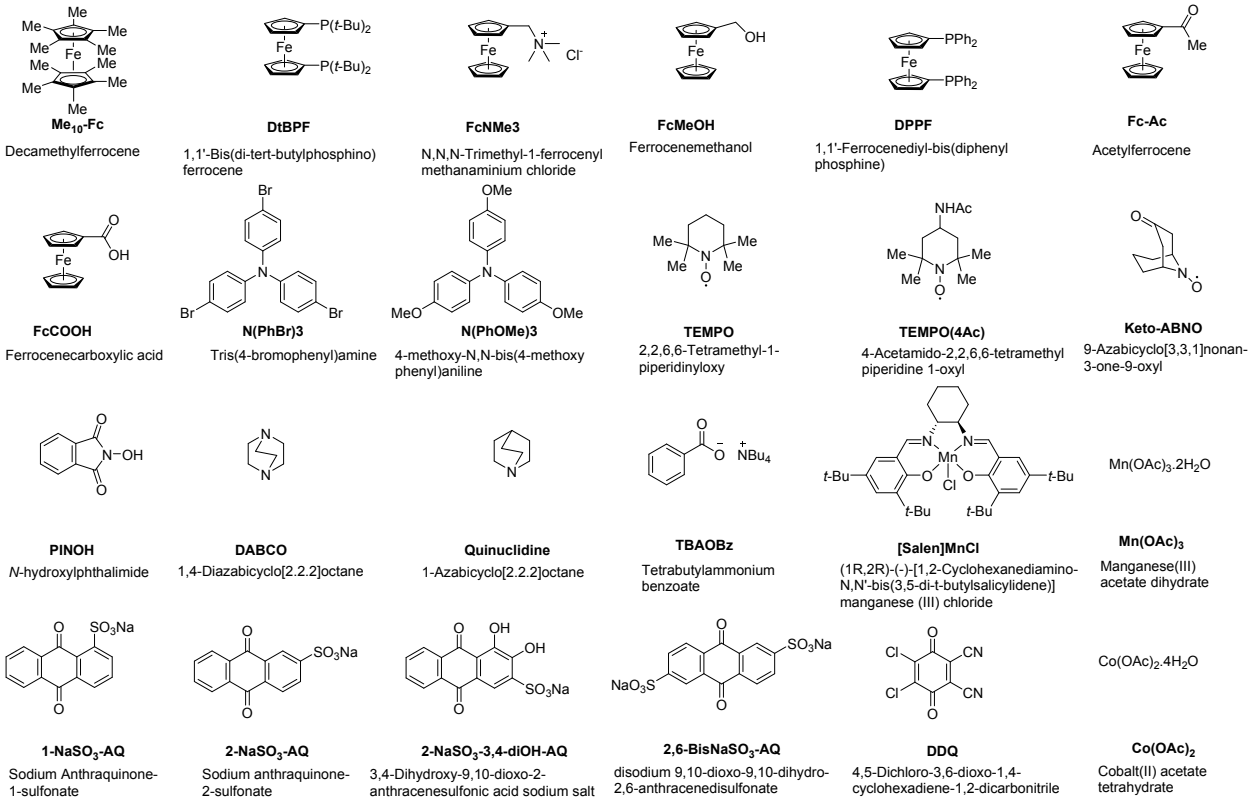
10.1. Redox mediator screening:



First, a stock solution of ADP was prepared as follows: 2.64 g (24 mmol, 2 eq) of sodium pyruvate was dissolved in ~100 mL of water. Potassium Phosphate dibasic (2.99 g, 13.2 mmol, 1.1 eq.), ADP disodium salt dihydrate (6.09 g, 12 mmol, 1 eq.) and MgCl2 (1.2 mL of 1M stock in water, 1.2 mmol, 0.12 eq) were then added and stirred until full dissolution. The pH of the solution was adjusted to 6.25 using 8M KOH. TPP (55 mg, 0.12 mmol, 0.01eq.) was then added and the pH was readjusted to 6.25. Water was added to reach a final volume of 120 mL, after which 100 µL of antifoam was added (Glanapon 2000, Bussetti & Co.)

Then, a stock of enzyme was prepared as follows: 8.4 mg of PO5 and 2 mg of AcK were dissolved in 0.2 mL of water (42 mg/mL PO5, 10 mg/mL AcK)

Finally, a 24-position aluminum well plate was plated with 1 mL glass vials pre-loaded with redox mediators (2 µmol per vial, see below for mediator list) and magnetic stir bars. The plate, the ADP and enzyme stock were then degassed and brought into a N_2 filled glovebox. 20 µL of DMSO was then added in the vials, followed by 400 µL of ADP stock and 4 µL of enzyme stock (final enzyme loading: 0.95 wt% PO (with respect to ADP) and 0.24 wt% AcK). The well plate was outfitted with the HT*e*-Chem[4] set up with graphite anodes and stainless steel cathodes. The reaction was stirred at room temperature at 800 rpm and 0.1 mA of current was passed for 19h. After that, samples were taken (30 µL of solution diluted with 500 µL of 4:1 Water:MeCN) in a 1 mL 96 well plate and the samples were centrifuged. The samples were then analyzed by HPLC with the method A.



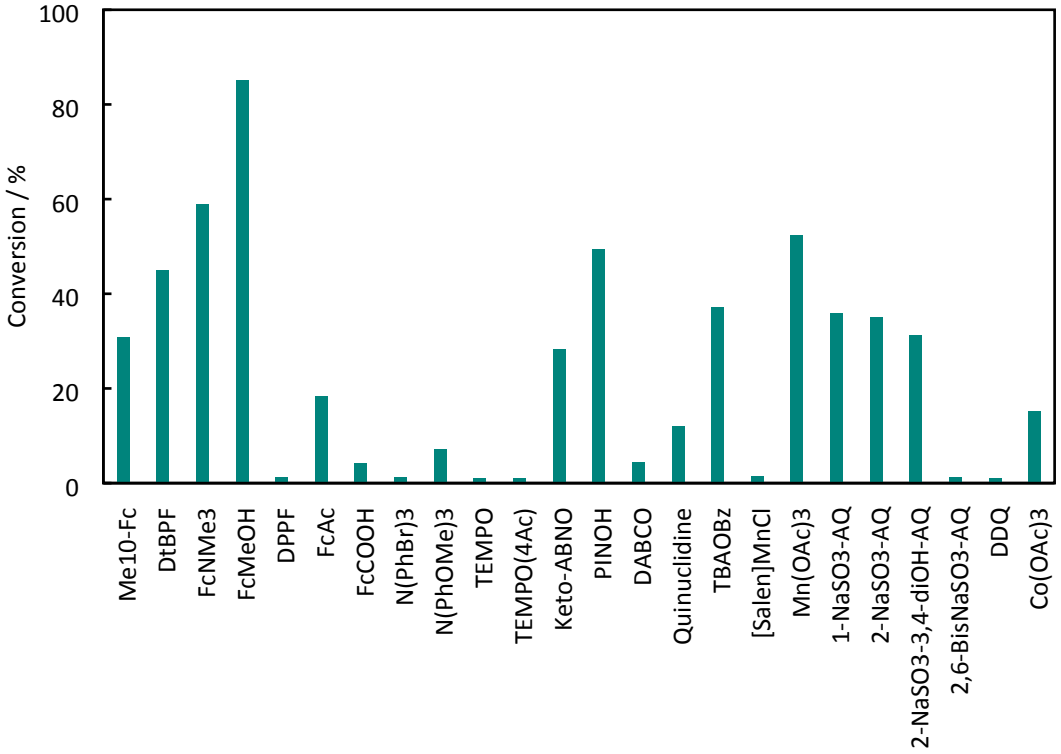
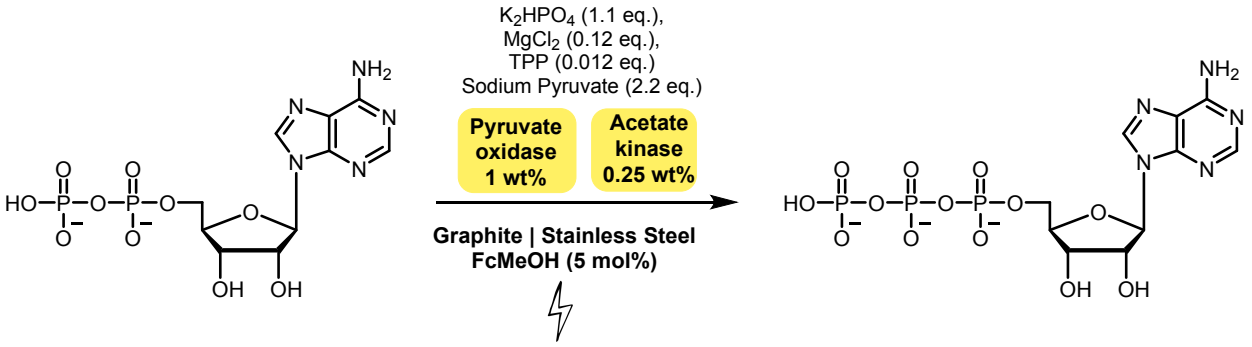


Figure S20: Structure of mediators screened. Screening results for the bioelectrochemical stoichiometric ATP formation. Each bar represents the conversion of ADP to ATP.

10.2. General procedure for bioelectrochemical stoichiometric ATP formation:



IKA Electrasyn scale: First, a stock solution of ADP and enzyme was prepared as described in 10.1. In a N_2 filled glovebox, a 5 mL electrasyn vial containing a magnetic stir bar was prepared by adding: 4.3 mg of FcMeOH, 0.2 mL of DMSO, 4 mL of ADP stock and 40 µL of enzyme stock (final enzyme loading: 0.95 wt% PO (with respect to ADP) and 0.24 wt% AcK). The vial was outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and placed on an electrasyn carousel and was stirred at 800 rpm at room temperature and the current was set to 1 mA. After 22h of passing constant current, the reaction was stopped and were sampled (20 µL of reaction was diluted with 500 µL of 4:1 Water/Acetonitrile mixture) and the samples were run with method A. The final conversion measured by LC was determined to be 89.4%

EasyMax scale:
86 mg of ferrocenemethanol (0.4 mmol, 0.05 eq.) was placed in a 100 mL EazyMax vessel and dissolved in 4 mL of DMSO. 80 mL of solution freshly prepared as described in section 10.1 was then added to the vessel. An easymax cap was then outfitted with an overhead stirrer, a temperature probe, a pH probe and a N_2 inlet, as well as 4 graphite electrodes (0.24"x6", McMaster-Carr, part #: 9121K71, polished with an 80-grit sand paper), 3 stainless steel electrodes (0.24", McMaster-Carr, part #: 89535K85) and a BASi RE-5B Ag/AgCl (3M NaCl) Reference Electrode (Part #:MF-2052), and placed on the vessel. The solution was then purged by bubbling N2 for 5 minutes and then maintaining a constant flow of N2 over the reaction and an overhead stirring of 700 rpm. Then, 15 mg of AcK101 (0.25 wt% with respect to ADP), and 60 mg of PO5 (1 wt% with respect to ADP) were charged via funnel. An EZsampler probe was then placed in the vessel and a sampling sequence was set to collect 12 samples over 30 h, with 80x dilution, diluted with a 4:1 water/MeCN solution. A constant current of 30 mA was passed for ~17.5 h at 25 °C. The current was stopped when the voltage sowed a sharp rise at ~16h indicating that the reaction was over (1740 C passed). The samples were analyzed by HPLC with method A. The final conversion measured by LC was determined to be 87.2%

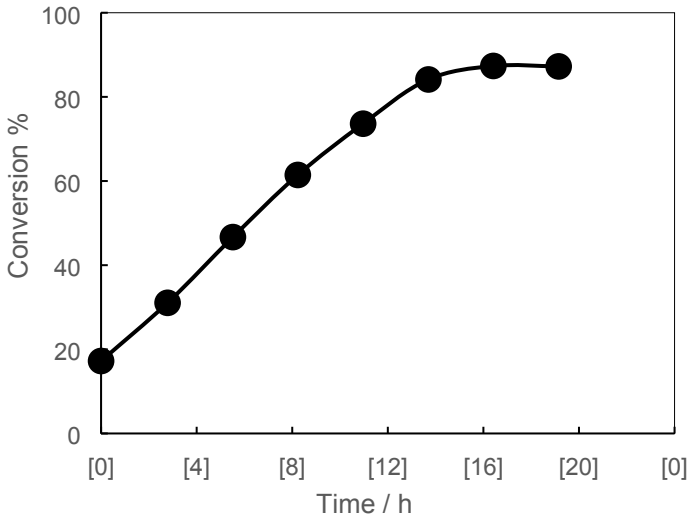


Figure S21: ADP to ATP conversion over time in the 80 mL (8.2 mmol) scale of the bioelectrochemical glycosylation. The conversion was calculated from the LC chromatograms of the EZsampler samples analyzed with method A.

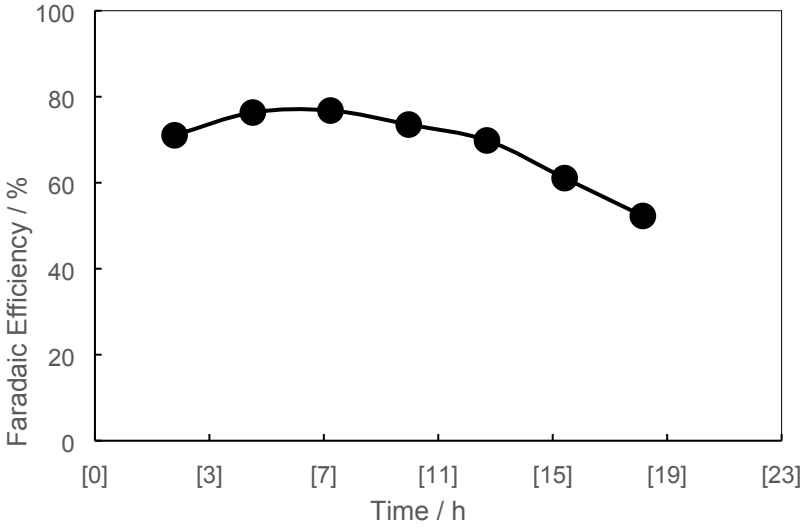


Figure S22: Faradaic efficiency over time in the 80 mL (8 mmol) scale of the bioelectrochemical ATP synthesis. The faradaic efficiency was calculated from the conversion and the charge passed at each time point.

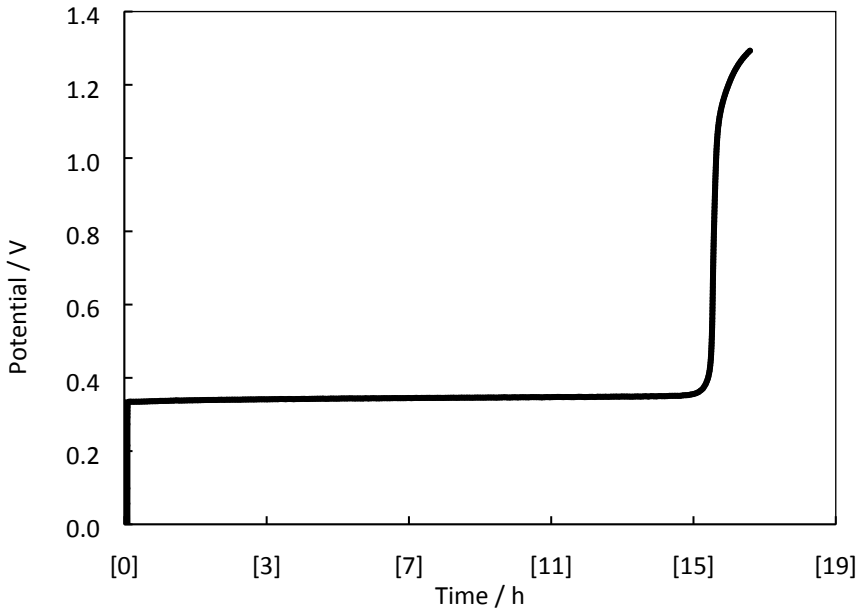


Figure S23: Voltage over time in the 80 mL (8 mmol) scale of the bioelectrochemical ATP synthesis.

10.3. Control experiments:

First, a stock solution of ADP and enzyme was prepared as described in 10.1. Then, in a N_2 filled glovebox, four 5 mL electrasyn vials containing a magnetic stir bar were prepared by adding:
a) No mediator control: 0.2 mL of DMSO, 4 mL of ADP stock and 40 µL of enzyme stock (final enzyme loading: 0.95 wt% PO (with respect to ADP) and 0.24 wt% AcK).
b) No PO control: 4.3 FcMeOH, 0.2 mL of DMSO, 4 mL of ADP stock and 0.4 mg of AcK (final enzyme loading: 0.25 wt% AcK).
c) No current control: 4.3 FcMeOH, 0.2 mL of DMSO, 4 mL of ADP stock and 40 µL of enzyme stock (final enzyme loading: 0.95 wt% PO and 0.24 wt% AcK),
d) air control: 4.3 FcMeOH, 0.2 mL of DMSO, 4 mL of ADP stock and 40 µL of enzyme stock (final enzyme loading: 0.95 wt% PO and 0.24 wt% AcK).
Each vial was outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode. Reaction a) and b) were placed on an electrasyn carousel and were stirred at 800 rpm and the current was set to 1 mA. Reaction dc) was stirred at 800 rpm under N_2 with no current, and reaction d) was brought outside of the glovebox and was stirred at 800 rpm in air. All reactions were run at room temperature. After 22h of passing constant current, the reactions were stopped and were sampled (20 µL of reaction was diluted with 500 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method A.

Table S3: Results for the bioelectrochemical stoichiometric ATP formation under different control conditions.

Conditions	*ATP conversion (%)*	*AMP conversion (%)*
no mediator	11.1	12.6
no PO	10.7	14.0
no current	21.5	24.6
with air, no catalase	33.9	9.4

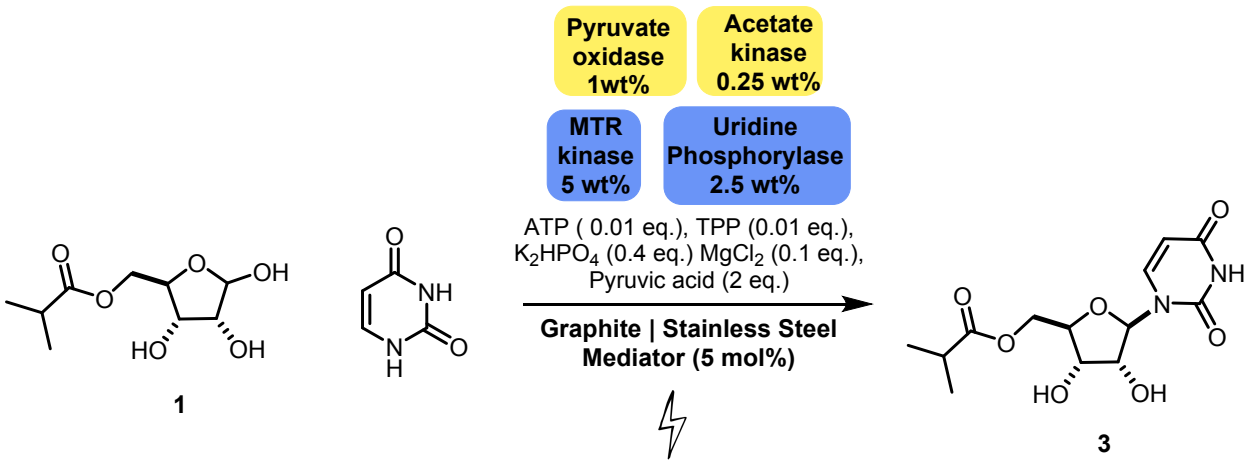
10.4. pH Study:

First, a stock solution of **1** and an enzyme stock prepared as described in 10.1. Then, in a N_2 filled glovebox, four 5 mL electrasyn vial containing a magnetic stir bar were prepared by adding: 4.3 mg of FcMeOH (0.02 mmol, 0.05 eq.) and 4 mL of ADP stock. Then, the pH of each vial was adjusted with either ~1M acetic acid or 8M KOH to have the following pH: 5, 6, 7, 8 (pH > 8 lead to precipitation of Mg salts). 40 µL of enzyme stock was added (final enzyme loading: 0.95 wt% PO (with respect to ADP), 0.24 wt% AcK). The vial was outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and was placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. After 24h of passing constant current, the reactions were stopped and were sampled (20 µL of reaction was diluted with 500 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method B.

Table S4: Results for the pH dependence on the bioelectrochemical ATP synthesis.

pH	*Conversion (%)*
5	0
6	81.7
7	67.3
8	64.8

11. Bioelectrochemical glycosylation



11.1. Redox mediator screening:

A stock solution of **1** was prepared as follows: in a 250 mL beaker, ~40 mL of water was added. 1.7 ml of pyruvic acid (24.52 mmol, 2 eq.) was added, followed by 0.86 g of potassium phosphate dibasic (4.9 mmol, 0.4 eq) and $MgCl_2$ (0.60 mmol, 1.20 mL of 1M stock in water, 0.1 eq.). Adjust the pH to ~6.0 with 8.9 M KOH. Then, 15.9 g of a 17 wt% solution of **1** (2.7 g, 12.26 mmol, 1 eq.). 74 mg of ATP (disodium hydrate salt, 0.123 mmol, 0.01 eq.) and 56 mg of TPP (0.123 mmol, 0.01 eq.). Adjust pH to 6.2 with KOH and adjust the volume to 120 mL by adding water. Add 25 µL of antifoam (Glanapon 2000 Konz).

An enzyme stock solution was prepared as follows: In a N_2 filled glovebox, 1.1 mg of AcK, 22.5 mg of MTR kinase, 11.1 mg of UP, 4.5 mg of PO_5 and was dissolved 1 mL of water.

Finally, a 24-position aluminum well plate was plated with 1 mL glass vials pre-loaded with redox mediators (2 µmol per vial, see below for mediator list) and magnetic stir bars. To 10 mL of the stock solution of **1** was added 108 mg of uracil. Then, the plate, the **1** stock solution and enzyme stock were then degassed and brought into a N_2 filled glovebox. 20 µL of DMSO was then added in the vial, followed by 400 µL of **1**+uracil stock and 20 µL of enzyme stock (final enzyme loading: 0.91 wt% PO (with respect to **1**) and 0.23 wt% AcK, 4.6 wt% MTR kinase, 2.3 % UP). The well plate was outfitted with the HT*e*-Chem[4] set up with graphite anodes and stainless steel cathodes. The reaction was stirred at room temperature at 800 rpm and 0.1 mA of current was passed for 24h. After that, samples were taken (20 µL of solution diluted with 500 µL of 4:1 Water:MeCN) and placed in a 1 mL 96 well plate and the samples were centrifuged. The samples were then analyzed by HPLC with the method B.

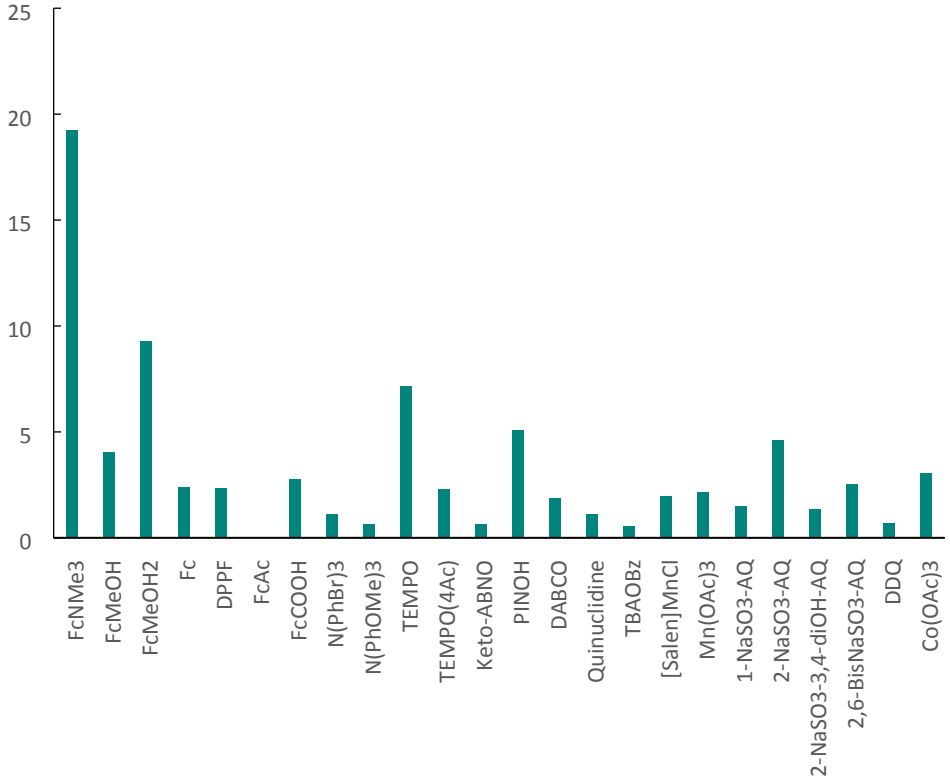
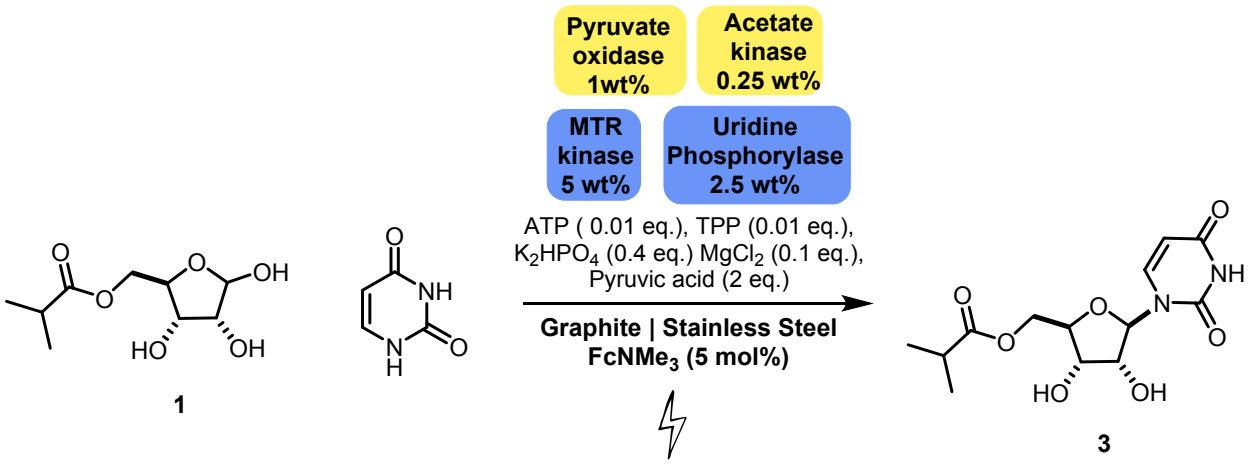


Figure S24: Screening results for the bioelectrochemical glycosylation. Each bar represents the conversion of uracil to product **3**.

11.2. General procedure:



IKA Electrasyn scale: First, a stock solution of **1** and an enzyme stock were prepared as described 11.1. Then, in a N_2 filled glovebox, a 5 mL electrasyn vial containing a magnetic stir bar was prepared by adding: 6 mg of FcNMe3 (0.02 mmol, 0.05 eq.), 46 mg of uracil (0.41 mmol, 1 eq.), 4 mL of **1** stock and 200 µL of enzyme stock (final enzyme loading: 0.95 wt% PO (with respect to **1**), 0.24 wt% AcK, 4.75 wt% MTR kinase, 2.4 wt% UP). The vial was outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and was placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. After 24h of passing constant current, the reactions were stopped and were sampled (20 µL of reaction was diluted with 500 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method A.

Chemspeed Swing: A stock solution of **1** was prepared as follows: in a 250 mL beaker, water ~20 mL was added, then 2.0 mL of pyruvic acid (28.6 mmol, 1.4 equiv), followed by 0.36 g K2HPO4 (2.0 mmol, 0.1 eq.), and MgCl2 (0.50 mmol, 0.5 mL of 1M stock in water, 0.025 eq.). The pH was adjusted to ~6.0 with 8 M KOH. 26 g of a 17.4 wt% aqueous solution of **1** (4.5 g, 20.4 mmol, 1 eq.), 62 mg of ATP (disodium hydrate salt, 0.10 mmol, 0.05 eq), 24 mg of TPP (0.05 mmol, 0.0025 eq.) and 300 mg of FcNMe3 (1.0 mmol, 0.05 eq) was added, then the pH was adjusted to 6.25 with 8M KOH and the volume was adjusted to 50 mL by adding water and 25 µL antifoam (Glanapon 2000 Konz). In a different vial a stock solution of enzymes was prepared: MTR kinase 180 mg, AcK 10 mg, PO 36 mg, UP 90 mg and 2 mL of water. Then, in a N_2 filled glovebox, six 5 mL Electrasyn vials containing a magnetic stir bar were prepared by adding: 183 mg of uracil (1.63 mmol, 1 eq.), 4 mL of **1** stock and 200 µL of enzyme stock and 200 µL of enzyme stock (final enzyme loading: 1 wt% PO (with respect to **1**), 0.25 wt% AcK, 5 wt% MTR kinase, 2.5 wt% UP). The vials were outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode, as well as an IKA Ag/AgCl wire reference electrode. Each vial was connected to a different MultiPalmSens4 channel via palmsens cables PALM-CBL-SNS5L4 that would contact the appropriate electrode on the electrasyn cap. The currents were set up in Multitrace to deliver 2, 2.5, 3, 3.5, 4 and 5 mA to each respective vial for 48 h. The vials were aged in a Chemspeed Swing robot outfitted with a V&P Scientific tumble stirrer, a 4 needle-head liquid handling tool, and a custom 6-well reactor block prepared for electrasyn vials. The robot agitated electrasyn vials at 500 rpm and sampled 14 µl from each vial into a 96-well polypropylene LC block containing 700 µl of 30% MeCN/H2O every 1 h. The samples were analyzed by HPLC with method A.

Table S5: Results for current screening the bioelectrochemical glycosylation

Conditions	*Conversion (%)*
2	73.2
2.5	62.2
3	57.6
3.5	50.3
4	49.1
5	39.4

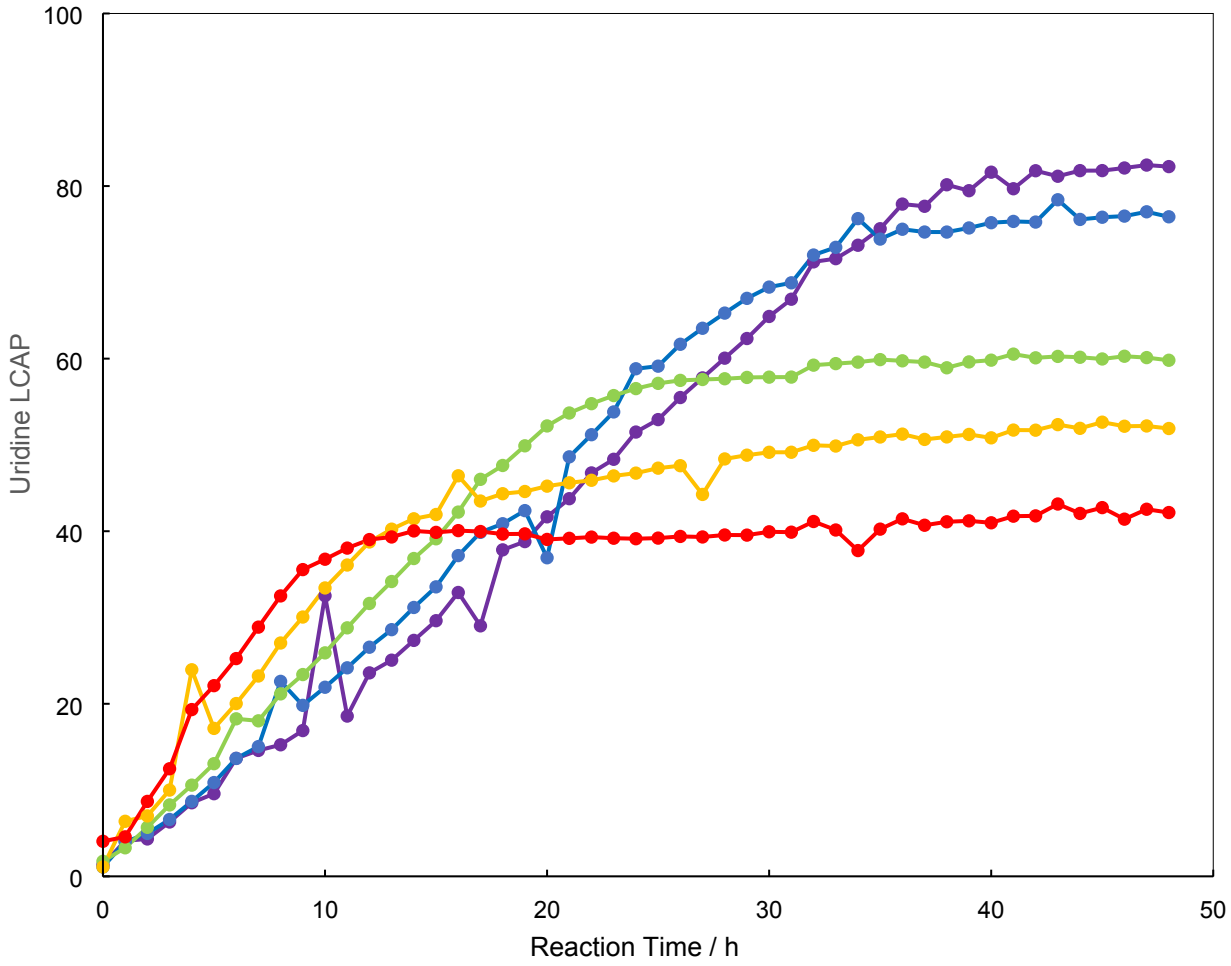


Figure S25: Kinetic trace for the formation of **3** under different currents: (—) 2 mA, (—) 2.5 mA, (—) 3 mA, (—) 4 mA, (—) 5 mA. The 3.5 mA experiment had a sampling issue and is therefore not shown here.

EasyMax scale: A stock solution of **1** was prepared as follows: in a 250 mL beaker, water (~20 mL) was added, then 3.41 mL of pyruvic acid (49 mmol, 2 eq.), followed by 0.43 g of K_2HPO_4 (2.5 mmol, 0.1 eq.), and MgCl2 (0.60 mmol, 0.6 mL of 1M stock in water, 0.025 eq.). Adjust pH to ~6.0 with 8 M KOH. 30 g of 18 wt% aqueous solution of **1** (5.4g, 24.5 mmol, 1 eq.), 148 mg of ATP (disodium hydrate salt, 0.25 mmol, 0.01 eq), 113 mg of TTP (0.25 mmol, 0.01 eq.). 360 mg of FcNMe3 (1.2 mmol, 0.05 eq) were added, then the pH was adjusted to 6.25 with 8M KOH and the volume was adjusted to 60 mL by adding water and 25 µL antifoam (Glanapon 2000 Konz).

In a 100 mL easymax vessel, 20 mL of **1** stock solution was diluted with 60 mL of 40 mM potassium phosphate and 10 mM $MgCl_2$, and the pH was adjusted to 6.25 with acetic acid. An easymax cap was then outfitted with an overhead stirrer, a temperature probe, a pH probe and a N_2 inlet, as well as 4 graphite electrodes (0.24"x6", McMaster-Carr, part #: 9121K71, polished with an 80-grit sand paper), 3 stainless steel electrodes (0.24", McMaster-Carr, part #: 89535K85) and a BASi RE-5B Ag/AgCl (3M NaCl) Reference Electrode (Part #:MF-2052), and placed on the vessel. The solution was then purged by bubbling N_2 for 5 minutes and then maintaining a constant flow of N_2 over the reaction. Then, 916 mg of uracil (8.2 mmol, 1 eq.) was added, followed by 4.5 mg of AcK (0.25 wt% with respect to **1**), 90 mg of MTR kinase (5 wt%), 45 mg of UP (2.5 wt%) and 18 mg of PO (1 wt%). An EZsampler probe was placed in the vessel and a sampling sequence was set to collect 12 samples over 33 h, with 80x dilution, diluted with a 4:1 water/MeCN solution. A constant current of 30 mA was passed for ~20 h at 25 °C, with a stirring of 700 rpm. After

a total of 2073 C was passed, the reaction was stopped. An NMR sample was taken by diluting 50 µL of reaction with 650 µL of D_2O. All the EZsampler samples were analyzed using the method B. The final uracil conversion was determined to be 96%, while no **1** was observed in the NMR spectrum.

The reaction was repeated at different currents. The reaction time was prolonged or shortened until conversion hit a plateau. Notably, for reactions that lead to lower conversions, the reaction mixture turns green when the conversion stops and the voltage goes up. This is due to the accumulation of oxidized ferrocene (deep blue color) in the reaction mixture.

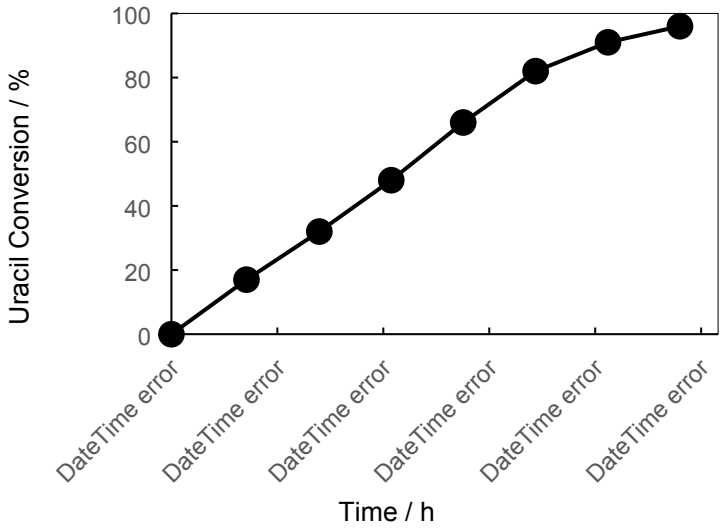


Figure S26: Uracil conversion over time in the 80 mL (8.2 mmol) scale of the bioelectrochemical glycosylation. The conversion was calculated from the LC chromatograms of the EZsampler samples analyzed with method B.

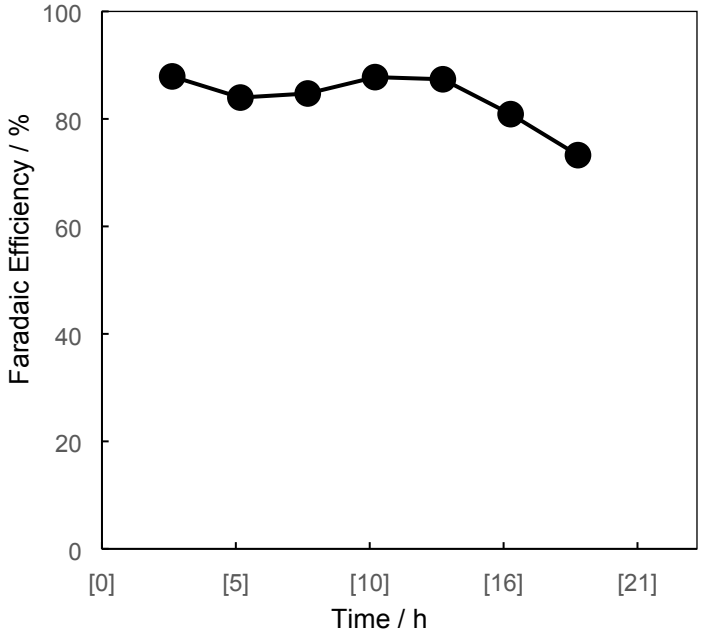


Figure S27: Faradaic efficiency over time in the 80 mL (8.2 mmol) scale of the bioelectrochemical glycosylation. The faradaic efficiency was calculated from the conversion and the charge passed at each time point.

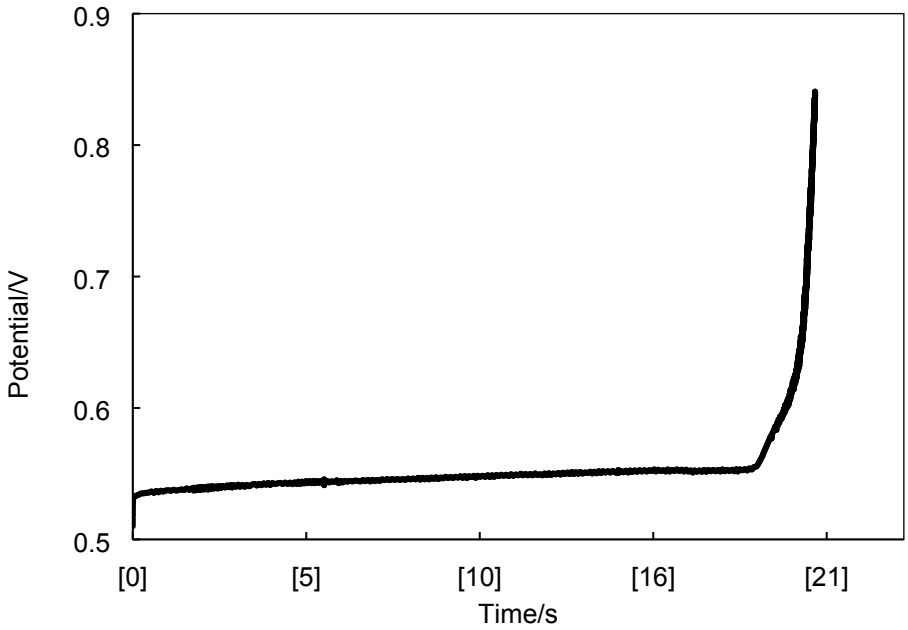


Figure S28: Voltage over time in the 80 mL (8.2 mmol) scale of the bioelectrochemical glycosylation.

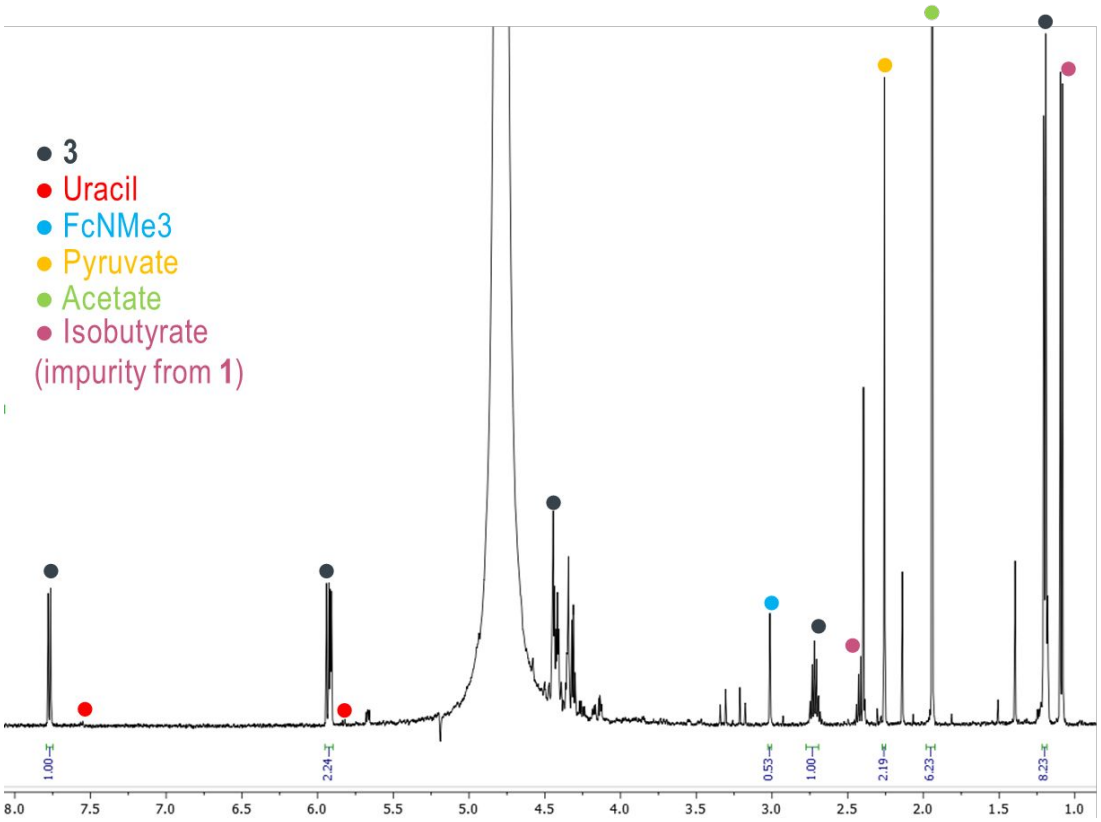


Figure S29: ^{1}H NMR spectrum in D_2O of the final reaction mixture of the 80 mL (8.2 mmol) scale of the bioelectrochemical glycosylation. Starting material **1** has peaks at 5.29 and 5.43 ppm.

Table S6: Current study for easymax scale reactions.

Current (mA)	*Current density (mA/cm²)*	*Concentration (g/L)*	*Time (h)*	*Acyl-ribose conversion NMR (%)*
10	0.4	22.5	65	99
20	0.8	22.5	26	99
30	1.2	22.5	20	99
40	1.6	22.5	<10	26
50	2	22.5	<10	56
45	1.8	45	24	99
60	2.4	45	10	58

Flow: A stock solution of **1** was prepared as follows: in a 1L glass bottle, water ~200 mL was added, then 22.5 g of sodium pyruvate (204 mmol, 2 eq.), followed by 7.11 g of K_2HPO_4 (40.8 mmol, 0.4 eq.), and MgCl2 (10.2 mmol, 10.2 mL of 1M stock in water, 0.1 eq.). Adjust pH to ~6.0 with 8 M KOH. 142 g of 15.8 wt% aqueous solution of **1** (22.4 g, 102 mmol, 1 eq.). Water was added to reach ~800 mL total volume, then 581 mg of ATP (disodium hydrate salt, 1.02 mmol, 0.01 eq), 470 mg of TTP (1.02 mmol, 0.01 eq.) were added. 4.49 g of FcNMe3 (15.3 mmol, 0.15 eq) was added, then the pH was adjusted to 6.25 with 8M KOH and the volume was adjusted to 80 mL by adding water and 120 µL antifoam (Glanapon 2000 Konz).

The stock solution was transferred to a 1000-mL OptiMax vessel on which was placed a vessel cover equipped with a Mettler Toledo React IR 45 probe and a Mettler Toledo FBRM probe, a Mettler Toledo EZsampler probe, a pH probe, a temperature probe and a N2 inlet. The solution was purged with N_2 for 30 minutes. Then, 11.43g of uracil (102 mmol, 1 eq.) was added, followed by 56 mg of AcK (0.25 wt% with respect to **1**), 1.13 g of MTR kinase (5 wt%), 560 mg of UP (2.5 wt%) and 225 mg of PO (1 wt%). The reactor contents were brought to 25 °C and the reaction mixture was recycled around the MP cell at 1200 mL/min. A constant current of 0.373 A (1.5 mA/cm², 3.65 A/mol) was applied and the sampling sequence was started. After ~20h, the sample analyzed with method B showed no further change in the uracil conversion (94%) and the reaction was stopped.

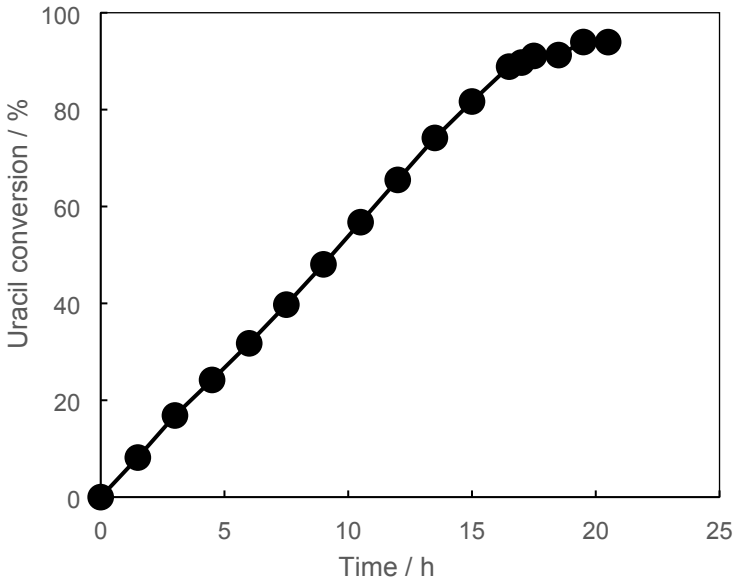


Figure S30: Uracil conversion over time in the 1 L (102 mmol) scale of the bioelectrochemical glycosylation in flow. The conversion was calculated from the LC chromatograms of the EZsampler samples analyzed with method B.

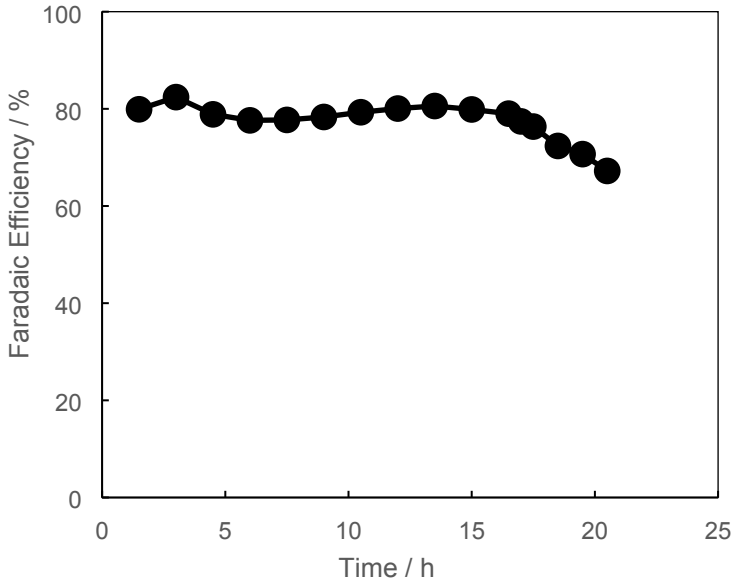


Figure S31: Faradaic efficiency over time in the 1 L (102 mmol) scale of the bioelectrochemical glycosylation in flow. The faradaic efficiency was calculated from the conversion and the charge passed at each time point.

11.3. Control experiments:

First, a stock solution of **1** prepared as described in 11.1. Then, 1.1 mg of AcK, 22.5 mg of MTR kinase 4BB, 11.1 mg of Uridine Phosphorylase 4BB were weighted out, brought in a glovebox and dissolved in 1 mL of water. In parallel, 4.5 mg of PO_5 was weighted out, brought in a glovebox and dissolved in 1 mL of water. Then, in a N_2 filled glovebox, four 5 mL electrasyn vials containing a magnetic stir bar were prepared by adding:

a) No mediator control: 46 mg of uracil (0.41 mmol, 1 eq.), 4 mL of **1** stock and 200 µL of enzyme stock and 200 µL of PO stock (final enzyme loading: 0.95 wt% PO (with respect to **1**), 0.24 wt% AcK, 4.75 wt% MTR kinase, 2.4 wt% UP).
b) No PO control: 6 mg of FcNMe3, 46 mg of uracil (0.41 mmol, 1 eq.), 4 mL of **1** stock and 200 µL of enzyme stock (final enzyme loading: 0.24 wt% AcK, 4.75 wt% MTR kinase, 2.4 wt% UP).
c) No current control: 6 mg of FcNMe3, 46 mg of uracil (0.41 mmol, 1 eq.), 4 mL of **1** stock and 200 µL of enzyme stock and 200 µL of PO stock (final enzyme loading: 0.95 wt% PO, 0.24 wt% AcK, 4.75 wt% MTR kinase, 2.4 wt% UP).
d) Air control: 6 mg of FcNMe3, 46 mg of uracil (0.41 mmol, 1 eq.), 4 mL of **1** stock and 200 µL of enzyme stock and 200 µL of PO stock (final enzyme loading: 0.95 wt% PO, 0.24 wt% AcK, 4.75 wt% MTR kinase, 2.4 wt% UP).
Each vial was outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode. Reaction a) and b) were placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. Reaction dc) was stirred at 800 rpm under N_2 with no current, and reaction d) was brought outside of the glovebox and was stirred at 800 rpm in air. All reactions were run at room temperature. After 24h of passing constant current, the reactions were stopped and were sampled (20 µL of reaction was diluted with 500 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method A.

Table S7: Results for the bioelectrochemical glycosylation under different control conditions.

Conditions	***Conversion (%)***
no mediator	2.0
no PO	1.3
No electricity	1.4
under air, no catalase	12.1

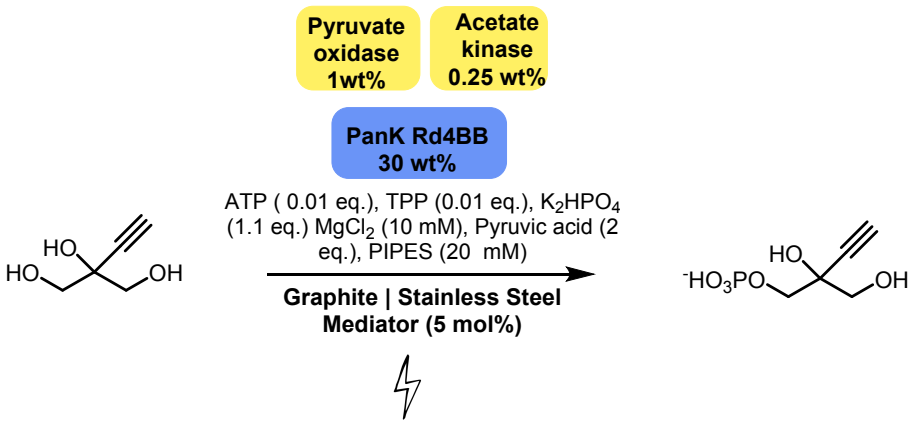
11.4. pH study:

First, a stock solution of **1** and an enzyme stock prepared as described in subsection 1: Redox mediator screening. Then, in a N_2 filled glovebox, four 5 mL electrasyn vial containing a magnetic stir bar were prepared by adding: 6 mg of FcNMe3 (0.02 mmol, 0.05 eq.), 46 mg of uracil (0.41 mmol, 1 eq.), 4 mL of **1** stock. Then, the pH of each vial was adjusted with either ~1M acetic acid or 8M KOH to have the following pHs: 4, 5, 6, 7, 8 (pH > 8 lead to precipitation of Mg salts). 200 µL of enzyme stock was added (final enzyme loading: 0.95 wt% PO (with respect to **1**), 0.24 wt% AcK, 2.4 wt% MTR kinase, 4.75 wt% UP). The vial was outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and was placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. After 24h of passing constant current, the reactions were stopped and were sampled (20 µL of reaction was diluted with 500 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method A.

Table S8: Results for the pH dependence on the bioelectrochemical glycosylation.

pH	***Conversion (%)***	***Uridine (Area %)***
4	0.3	0
5	3.1	0.2
6	86.8	0.3
7	61.9	0.7
8	87.2	1.9

12. Reaction scope:
12.1. 2-ethynylglycerol phosphorylation:



In a 100 mL flask, add water (~10 mL), followed by pyruvic acid (0.278 ml, 4 mmol), potassium phosphate dibasic (0.383 g, 2.2 mmol), MgCl2 (200 µL of 1M solution in water) and adjust pH to ~6.4 with 8 M KOH. Then add 2-ethynylglycerol (0.232 g, 2 mmol, 2.6 g of 8.86 wt% solution) followed by ATP (0.011 g, 0.02 mmol), TPP (9.22 mg, 0.02 mmol) and PIPES (0.121 g, 0.4 mmol). Adjust pH to ~7.5 with 8M KOH and adjust the volume to 20 mL by adding water. Weight out 2.32 mg of PO5 and 0.8 mg of AcK101 and 80 mg of PanK Rd4BB and dissolve in 1 mL of water. (2.32 mg/mL PO, 0.8 mg/mL AcK101, 80 mg/mL PanK).

a) Bioelectrochemical reaction: In two 5 mL electrasyn vials was added 4 mL of 2-ethynylglycerol stock solution and either FcMeOH (4.3 mg, 0.020 mmol) in 40 µL of DMSO or FcNMe3 (5.9 mg, 0.020 mmol) and the vials were brought inside a N_2 filled glovebox, after which 200 µL of enzyme stock was added. The vials were outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and were placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. After 44h of passing constant current, the reactions were stopped and sampled by diluting 50 µL of reaction with 600 µL of D_2O. The conversion was determined by integrating the ethynyl peak at 2.94 (SM) and 2.95 (product) ppm in the ^{1}H NMR spectra. The conversion of the reaction using FcMeOH and FcNMe3 was 79% and 82%, respectively.

b) O_2 and catalase reaction: in a 5 mL vial was added 1 mg of catalase (Roche), 4 mL of 2-ethynylglycerol stock solution and 200 µL of enzyme stock. The reaction was stirred at 800 rpm in air without a cap. After 24h the reaction was stopped and sampled by diluting 50 µL of reaction with 600 µL of D_2O. The conversion was determined by integrating the ethynyl peak at 2.94 (SM) and 2.95 (product) ppm in the ^{1}H NMR spectrum. The conversion of the reaction was 85%. Further sampling at 44h showed decreased conversion

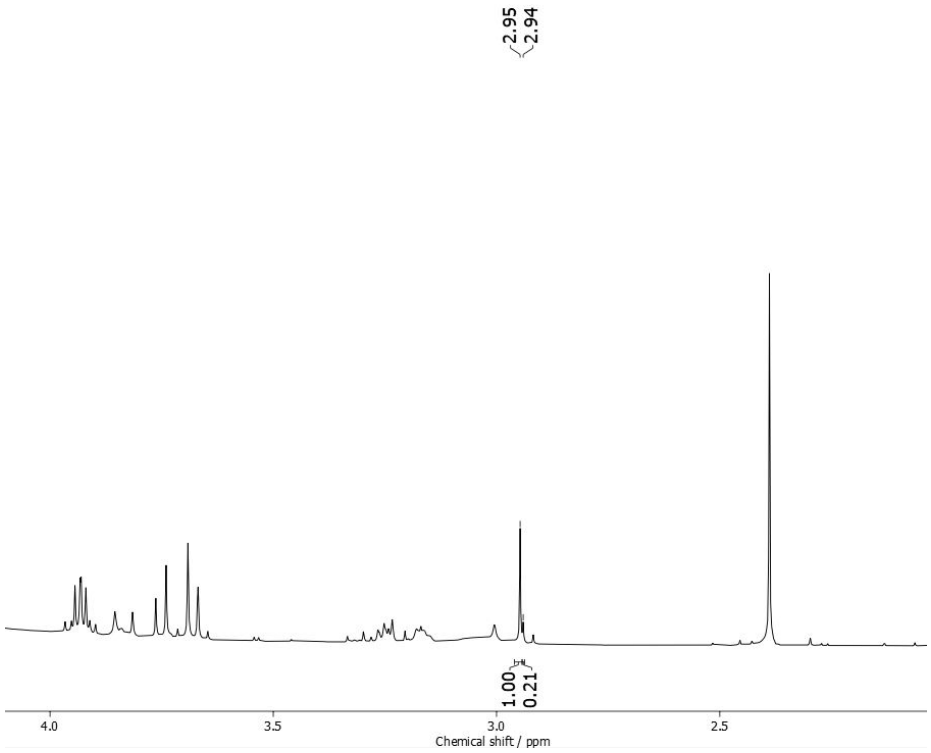


Figure S32: ^{1}H NMR spectrum of the reaction mixture of the bioelectrochemical phosphorylation of 2-ethynylglycerol using FcNMe3 as the mediator.

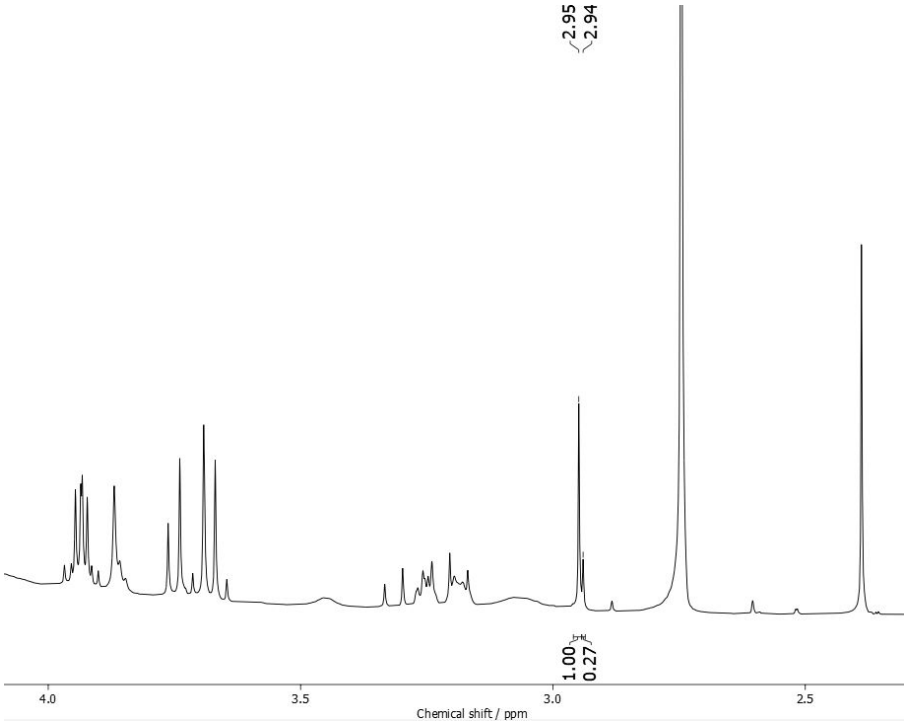


Figure S33: ^{1}H NMR spectrum of the reaction mixture of the bioelectrochemical phosphorylation of 2-ethynylglycerol using FcMeOH as the mediator.

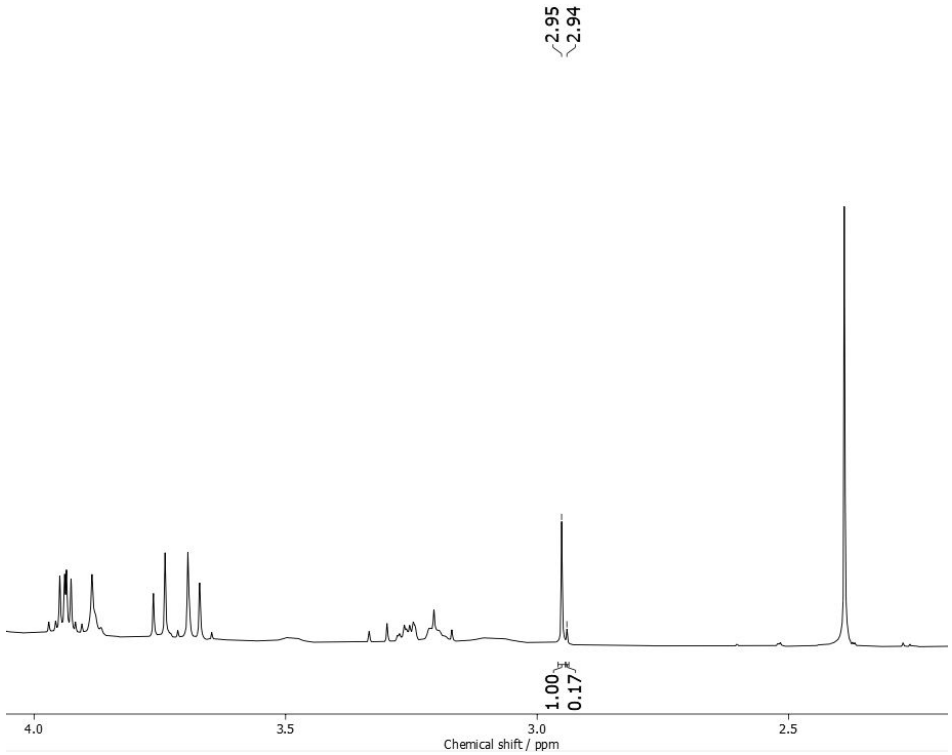
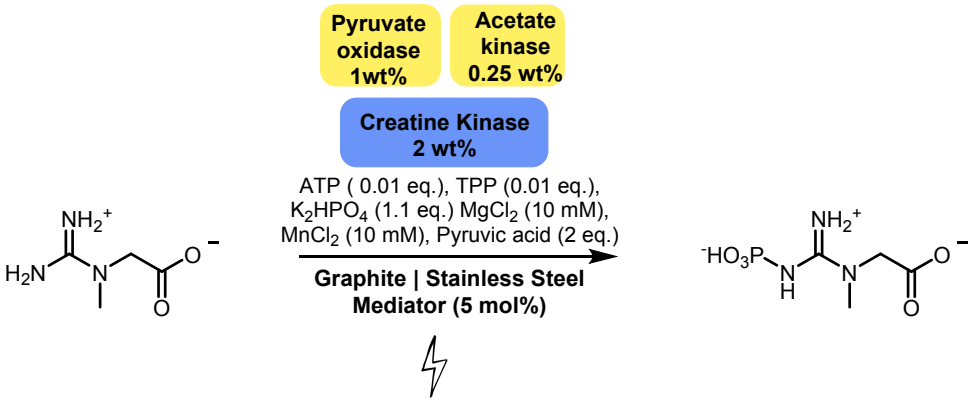


Figure S34: ^{1}H NMR spectrum of the reaction mixture of the bioelectrochemical phosphorylation of 2-ethynylglycerol with O_2 as the driving force in the presence of catalase.

12.2. Creatine phosphorylation:



In a 100 mL beaker, add water (~10 mL), followed by pyruvic acid (0.139 ml, 2 mmol), potassium phosphate dibasic (0.209 g, 1.2 mmol), MgCl2 (200 µL of 1M solution in water), $MnCl_2.4H_2O$ (4 mg, 0.02 mmol) and adjust pH to ~7.5 with 8 M KOH. Then add creatine (0.131 g, 1 mmol) followed by ATP (5.7 mg, 0.01 mmol), TPP (4.6 mg, 0.01 mmol). Adjust pH to ~7.5 with KOH and adjust the volume to 20 mL by adding water. Weight out 1.3 mg of PO5 and 0.3 mg of AcK101 and 2.6 mg of CK and dissolve in 1 mL of water. (1.3 mg/mL PO, 0.3 mg/mL AcK101, 2.6 mg/mL CK).

a) Bioelectrochemical reaction: In two 5 mL electrasyn vials was added 4 mL of creatine stock solution and either FcMeOH (4.3 mg, 0.020 mmol) in 40 µL of DMSO or FcNMe3 (5.9 mg, 0.020 mmol) and the vials were brought inside a N_2 filled glovebox, after which 200 µL of enzyme stock was added. The vials were outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and were

placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. After 24h of passing constant current, the reactions were stopped and sampled by diluting 50 µL of reaction with 600 µL of D_2O. The conversion was determined by integrating the peaks at 3.95 (SM, 2H) and 3.97 (product, 2H) ppm in the ^{1}H NMR spectra. The conversion of the reaction using FcMeOH and FcNMe3 was 26% and 7%, respectively.

b) O_2 and catalase reaction: in a 5 mL vial was added 1 mg of catalase (Roche), 4 mL of creatine stock solution and 200 µL of enzyme stock. The reaction was stirred at 800 rpm in air without a cap. After 24h the reaction was stopped and sampled by diluting 50 µL of reaction with 600 µL of D_2O. The conversion was determined by integrating the peaks at 3.95 (SM, 2H) and 3.97 (product, 2H) ppm in the ^{1}H NMR spectrum. The conversion of the reaction was 12%.

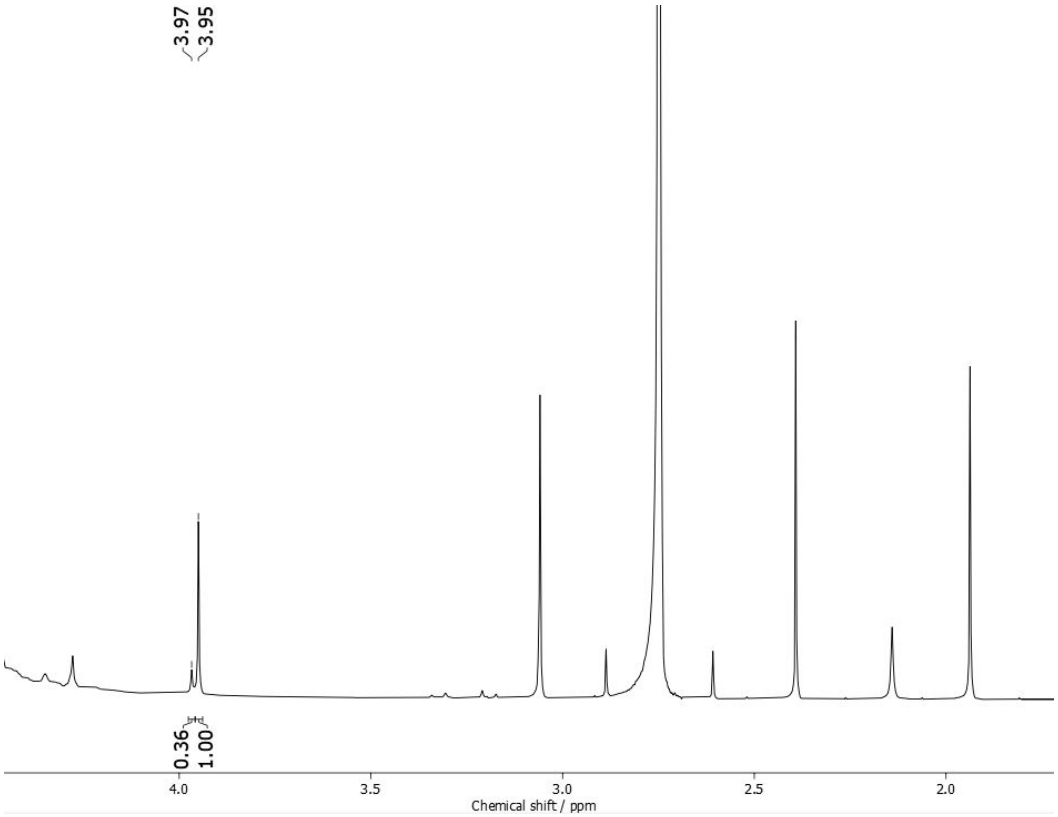


Figure S35: ^{1}H NMR spectrum of the reaction mixture of the bioelectrochemical phosphorylation of creatine using FcMeOH as the mediator.

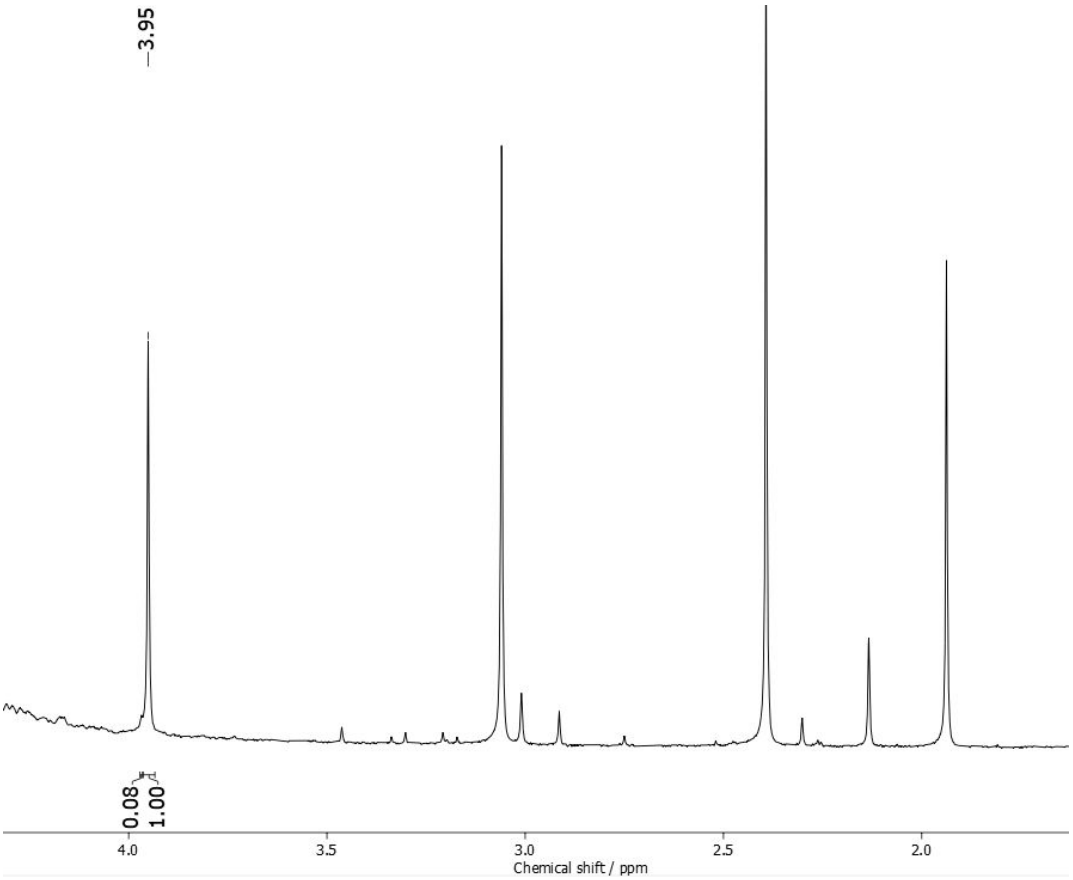


Figure S36: ^{1}H NMR spectrum of the reaction mixture of the bioelectrochemical phosphorylation of creatine using FcNMe3 as the mediator.

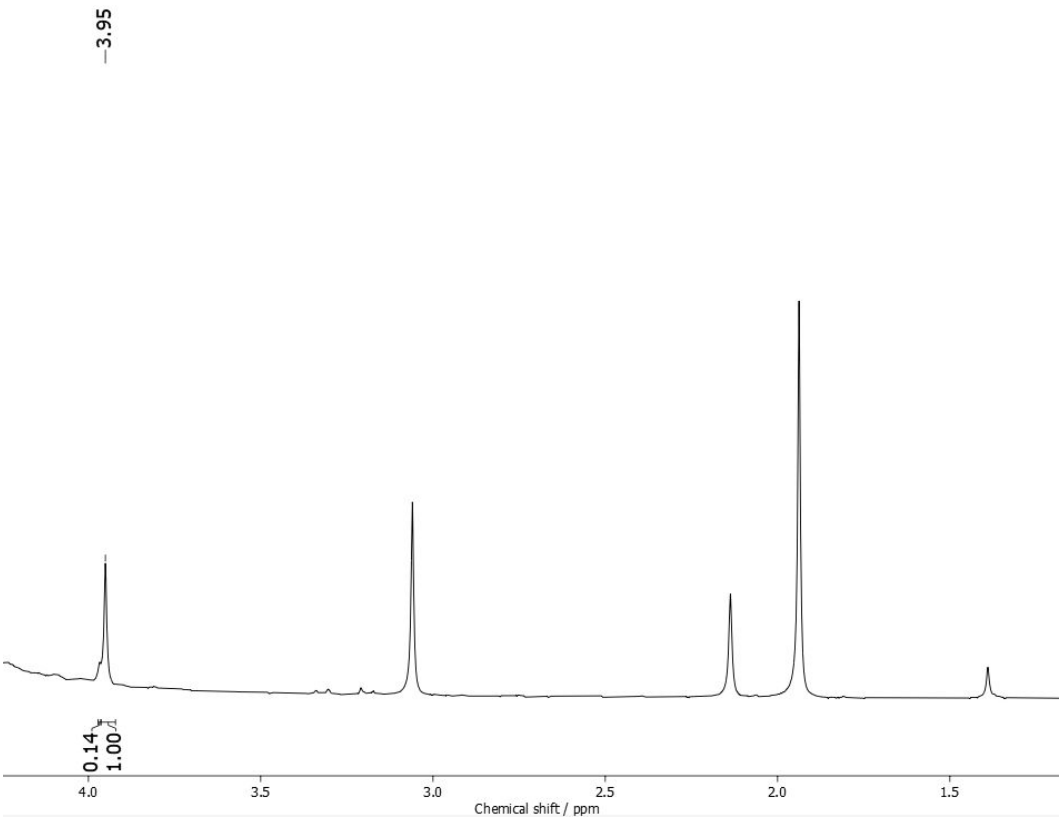
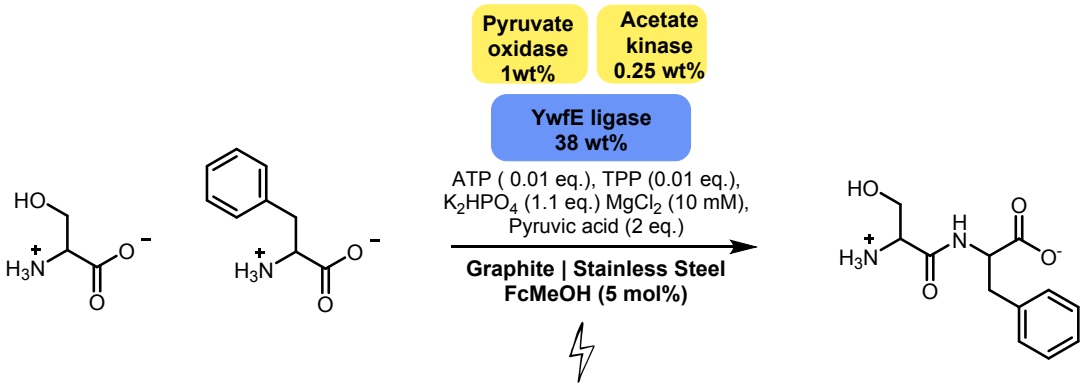


Figure S37: ^{1}H NMR spectrum of the reaction mixture of the bioelectrochemical phosphorylation of creatine with O_2 as the driving force in the presence of catalase.

12.3. Phe-Ser coupling:



In a 100 mL beaker, add water (~10 mL), followed by pyruvic acid (0.139 ml, 2 mmol), potassium phosphate dibasic (0.192 g, 1.1 mmol), MgCl2 (200 µL of 1M solution in water), and adjust pH to ~9 with 8 M KOH. Then add L-phenylalanine (0.165 g, 1 mmol), L-serine (0.105 g, 1 mmol) followed by ATP (5.7 mg, 0.01 mmol), and TPP (4.6 mg, 0.01 mmol). Adjust pH to 8 with KOH and adjust the volume to 20 mL by adding water. Weight out 1.1 mg of PO5 and 0.3 mg of AcK101 and dissolve in 1 mL of water. (1.1 mg/mL PO, 0.3 mg/mL AcK101).

a) Bioelectrochemical reaction: In two 5 mL electrasyn vials was added 8 mg of YwfE ligase, 4 mL of amino acid stock solution, and FcMeOH (4.3 mg, 0.020 mmol) in 40 µL of DMSO and the vial was brought inside a N_2 filled glovebox, after which 200 µL of enzyme stock was added. The vial was outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and was placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. After 40 h of passing

constant current, the reactions were stopped and sampled by diluting 20 µL of reaction with 400 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method C. The conversion of Phe to Phe-Ser was 35%.

b) O_2 and catalase reaction: in a 5 mL vial was added 8 mg of YwfE ligase, 1 mg of catalase (Roche), 4 mL of amino acid stock solution and 200 µL of enzyme stock. The reaction was stirred at 800 rpm in air without a cap. After 24h the reaction was stopped and sampled by diluting 20 µL of reaction with 400 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method C. The conversion was 5%. Further sampling up to 5 days showed decreased conversion

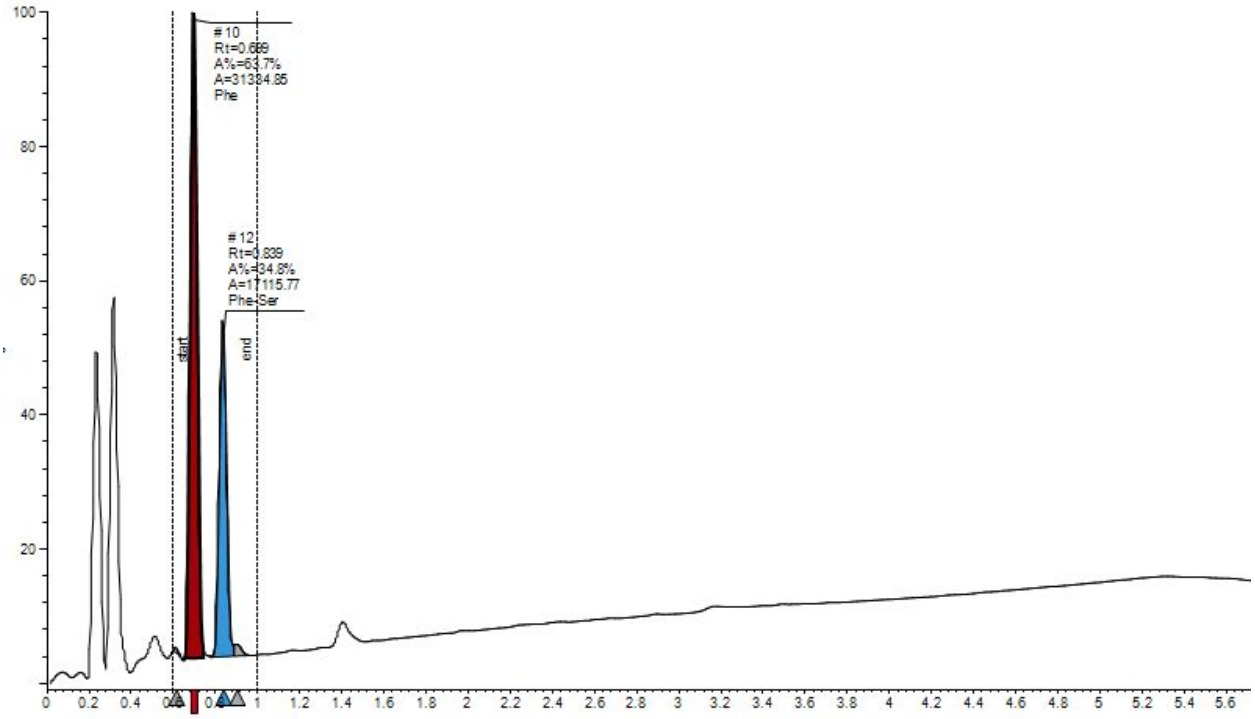


Figure S38: Chromatogram of the reaction mixture of the bioelectrochemical amino-acid coupling using FcMeOH as the mediator.

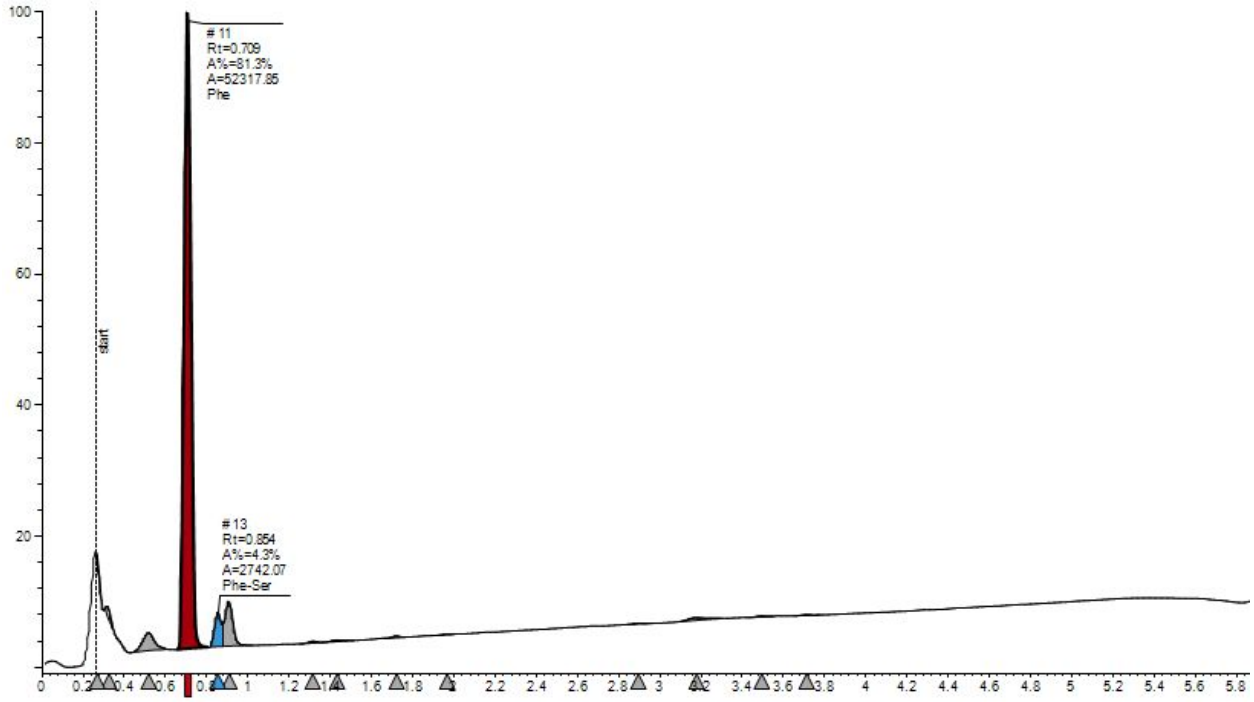
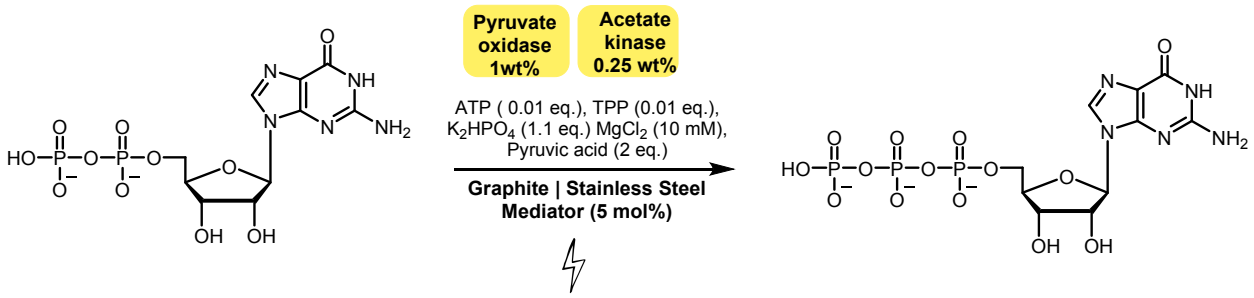


Figure S39: Chromatogram of the reaction mixture of the bioelectrochemical amino-acid coupling using O_2 as the driving force in the presence of catalase.

12.4. GDP phosphorylation:



In a 100 mL beaker, add water (~10 mL), followed by sodium pyruvate (0.44 g, 4 mmol), potassium phosphate dibasic (0.383 g, 2.2 mmol), MgCl2 (200 µL of 1M solution in water), and adjust pH to ~6.2 with 8 M KOH. Then add GDP trisodium salt (1.02 g, 2 mmol) and TPP (9.2 mg, 0.02 mmol). Adjust pH to 6.2 with KOH and adjust the volume to 20 mL by adding water. Weight out 10 mg of PO5 and 2.5 mg of AcK101 and dissolve in 1 mL of water. (10 mg/mL PO, 2.5 mg/mL AcK101).

a) Bioelectrochemical reaction: In two 5 mL electrasyn vials was added 4 mL of GDP stock solution and either FcMeOH (4.3 mg, 0.020 mmol) in 40 µL of DMSO or FcNMe3 (5.9 mg, 0.020 mmol) and the vials were brought inside a N_2 filled glovebox, after which 200 µL of enzyme stock was added. The vials were outfitted with an electrasyn cap with an IKA graphite anode and an IKA stainless steel cathode and were placed on an electrasyn carousel and was stirred at 800 rpm and the current was set to 1 mA. After 24h the reaction was stopped and sampled by diluting 20 µL of reaction with 400 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method A. The conversion of the reaction using FcMeOH and FcNMe3 was 88% and 59%, respectively.
b) O_2 and catalase reaction: in a 5 mL vial was added 1 mg of catalase (Roche), 4 mL of GDP stock solution and 200 µL of enzyme stock. The reaction was stirred at 800 rpm in air without a cap. After 24h the reaction

was stopped and sampled by diluting 20 µL of reaction with 400 µL of 4:1 Water/Acetonitrile mixture) and the samples were analyzed by HPLC with method A. The conversion was 85%.

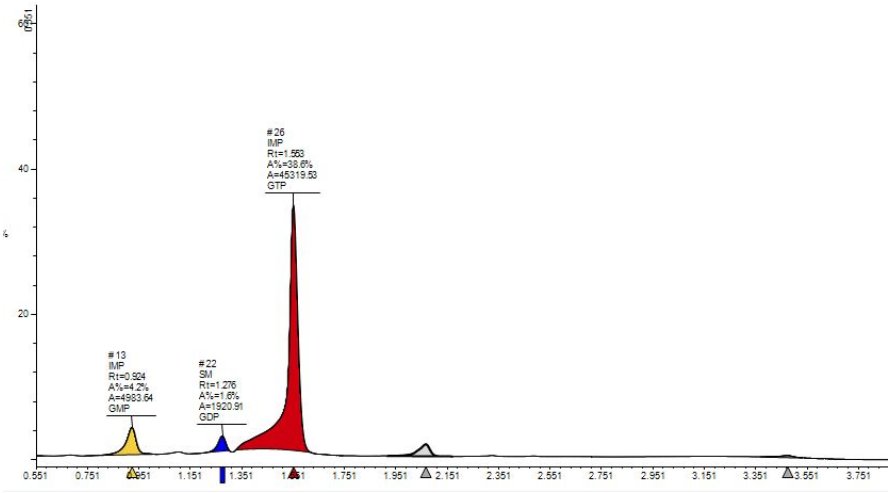


Figure S40: Chromatogram of the reaction mixture of the bioelectrochemical GDP phosphorylation using FcMeOH as the mediator.

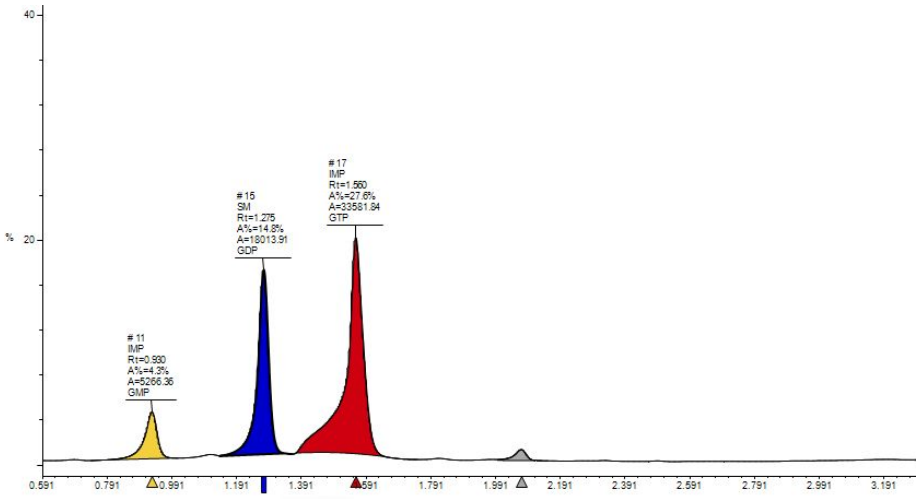


Figure S41: Chromatogram of the reaction mixture of the bioelectrochemical GDP phosphorylation using FcNMe3 as the mediator.

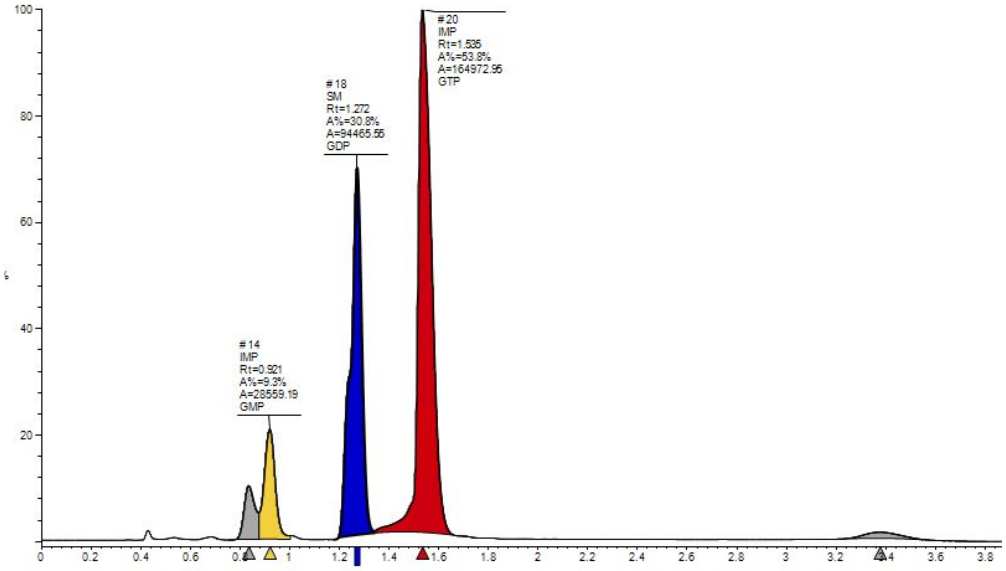


Figure S42: Chromatogram of the reaction mixture of the bioelectrochemical GDP phosphorylation using O_2 as the driving force in the presence of catalase.

13. DNA sequences of the enzymes:

PO

ATGACCCAGGGTAAAATTACCGCAAGTGCCGCAATGCTGAATGTGCTGAAAACCTG
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MTR Kinase
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UP
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GCGCGTCGTCTGCTG

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