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Transforming an esterase into an enantioselective catecholase through bioconjugation of a versatile metal-chelating inhibitor

Laura Fernandez-Lopez,[†a] Isabel Cea-Rama,[†b] Julia Alvarez-Malmagro,[†a] Anna K. Ressmann,[c] Jose L. Gonzalez-Alfonso,[a] Cristina Coscolín,[a] Patrick Shahgaldian,[d] Francisco J. Plou,[a] Jan Modregger,[c] Marcos Pita,[a] Julia Sanz-Aparicio,*[b] and Manuel Ferrer*[a]

Abstract: Metal complexes can be introduced into protein scaffolds to generate versatile biomimetic catalysts with a variety of catalytic properties. Here, we synthesized and covalently bound a bipyridinyl derivative to the active centre of an esterase to generate a biomimetic catalyst which shows catecholase activity and enantioselective catalytic oxidation of (+)-catechin.

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Experimental Procedures

Enzyme source, production and purification. The vector pBXNH3 and the host *Escherichia coli* MC1061 were the sources of His_6-tagged EH_3 (GenBank acc. nr. KY483645), which was produced and purified at 4°C (>98%) after binding to a Ni-NTA His-Bind resin (from Merck Life Science S.L.U., Madrid, Spain) as described previously.[S1] The ester hydrolase EH_{1AB1} (Protein data Bank (PDB) acc. nr. 6RKY) from metagenomic origin, was used for comparative studies; the vector and the source of the His_6-tag protein as well as the Ni-NTA His-Bind purification protocol (purity > 98%), were as described previously.[S2,S3]

Synthesis and characterization of inhibitor 2, 6-hexyl-[1,3,2]dioxaphosphepino[5,4-b:6,7-b']dipyridine 6-oxide. All reagents were purchased from commercial suppliers and used without further purification unless noted otherwise. Reagents were bought from Merck Life Science S.L.U. (Madrid, Spain), except 2,2'-bipyridine-3,3'-diol that was purchased from Carbosynth (Berkshire, UK), and solvents that were purchased from Carl Roth (Graz, Austria). For the synthesis of the suicide inhibitor **2**, dry glassware was used and the reaction was performed under Argon atmosphere. In a 4 ml vial 2,2'-bipyridine-3,3'-diol (166.8 mg, 0.89 mmol, 0.9 equiv.) was suspended in N-N-dimethylformamide (DMF) (1400 µl) and N,N-diisopropylethylamine (DIPEA) (603 µl, 3.55 mmol, 3.6 equiv.). The reaction was cooled to 0 °C and hexylphosphonic dichloride (200 mg, 0.98 mmol, 1 equiv.) was added. The color changed from turbid yellow to clear red. The reaction was allowed to warm up to room temperature after 30 min. After 180 min additional hexyl phosphonium dichloride (0.2 equiv.) was added and stirring continued overnight. High performance liquid chromatography–mass spectrometry (HPLC-MS) was used for reaction control, for which samples were diluted (1:40) using acetonitrile (ACN). The reaction mixture was extracted with hexane and hexane phase was discarded. The mixture was extracted three times with methyl tert-butyl ether (MTBE), the fractions were kept separately. The precipitated product was filtered off and washed with small portions of cold MTBE. The crystals were redissolved in MTBE and filtered over a small batch of silica gel. The solvent was evaporated *in vacuo* and a yellow solid was obtained. Yield: 55 mg (20%) yellow solid.

HPLC-MS samples were analyzed on a Waters Alliance HPLC equipped with a UV detector and an Electrospray Ionisation Mass Spectrometry (ESI-MS) detector (Acquity QDA). Samples were separated on a C18 column (Phenomenex; 0D-4462-EO, 2.6 µm; 4.6 x 100 mm) at 40 °C, detection was performed at 254 nm. A gradient of water (0.1% formic acid (FA), solvent A) and acetonitrile (0.1% FA, solvent B) was used (Table S1).

Table S1. Gradient used for HPLC detection used for the first experiments

Time [min]	Solvent A H_2O (0.1% FA) [%]	Solvent B ACN (0.1% FA) [%]	Flow [ml/min]
0	75	25	0.5
3	75	25	0.5
10	5	95	0.5
11	5	95	0.5
13	0	100	0.5
14	0	100	0.5
19	75	25	0.5
20	75	25	0.5

^{1}H (Figure S1), ^{13}C (Figure S2) and ^{31}P (Figure S3) NMR spectra were recorded from methylene chloride-d_2 solutions on a Bruker Avance III HD 600 (600 MHz) spectrometer by TU Wien. Chemical shifts (δ) are reported in ppm using tetramethylsilane as internal standard coupling constants (J) are in Hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet.

^{1}H NMR (600 MHz, Methylene Chloride-d_2) δ 8.73 (dt, *J* = 4.6, 1.2 Hz, 2H), 7.63 (dt, *J* = 8.2, 1.4 Hz, 2H), 7.49 (ddd, *J* = 8.2, 4.6, 0.6 Hz, 2H), 2.07 (ddd, *J* = 17.7, 9.0, 7.2 Hz, 2H), 1.82 – 1.75 (m, 2H), 1.50 – 1.43 (m, 2H), 1.32 (pt, *J* = 8.5, 3.6 Hz, 5H), 0.92 – 0.86 (m, 3H).

^{13}C NMR (150 MHz, CD_2Cl2) δ 148.40, 148.39, 146.84, 146.77, 146.63, 129.79, 129.77, 125.93, 31.70, 30.65 (d, J = 16.3 Hz, 24.31 (d, J = 132.7 Hz), 22.91, 22.53 (d, *J* = 5.4 Hz), 14.30.

^{31}P NMR (Methylene Chloride-d_2) δ 42.14.

2D-NMR experiments (HSQC und HMBC) were used for structure evaluation.

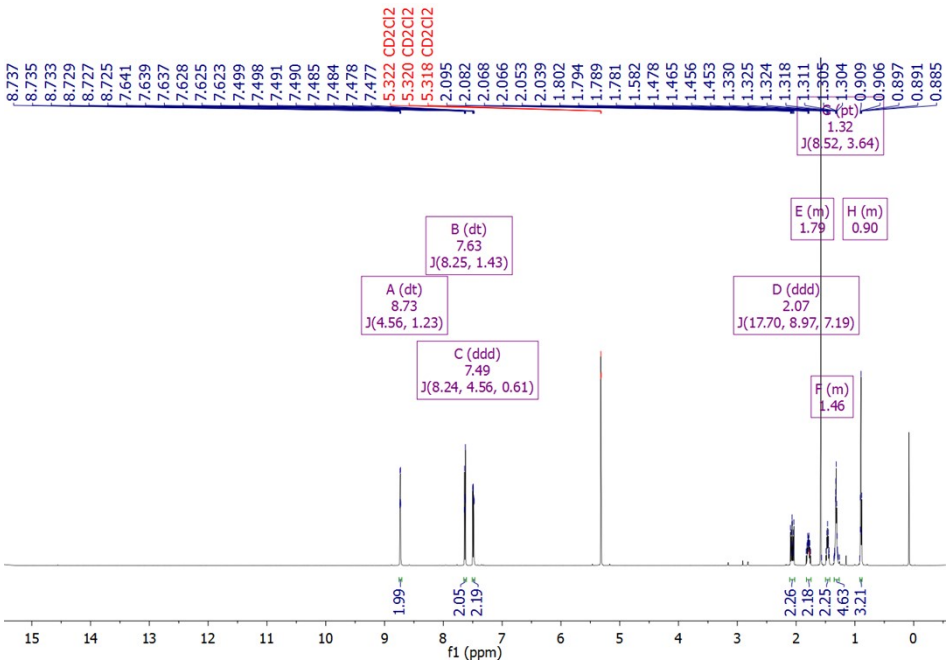

Figure S1. ^{1}H spectrum

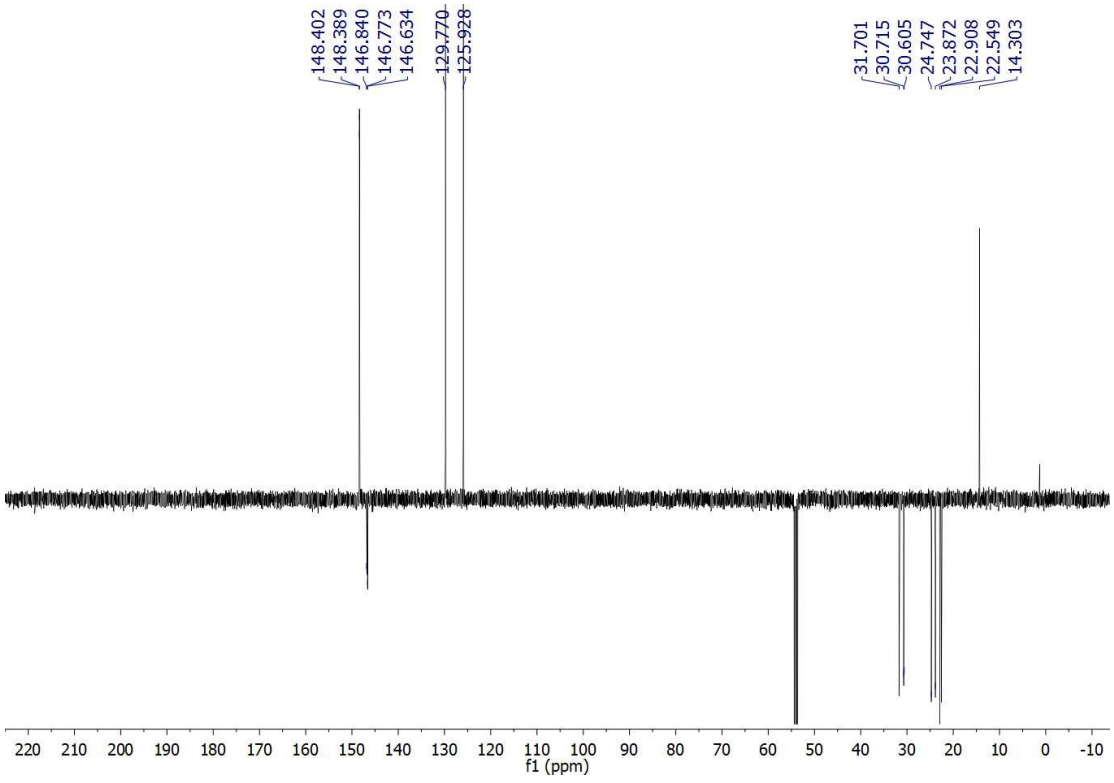

Figure S2. ^{13}C spectrum

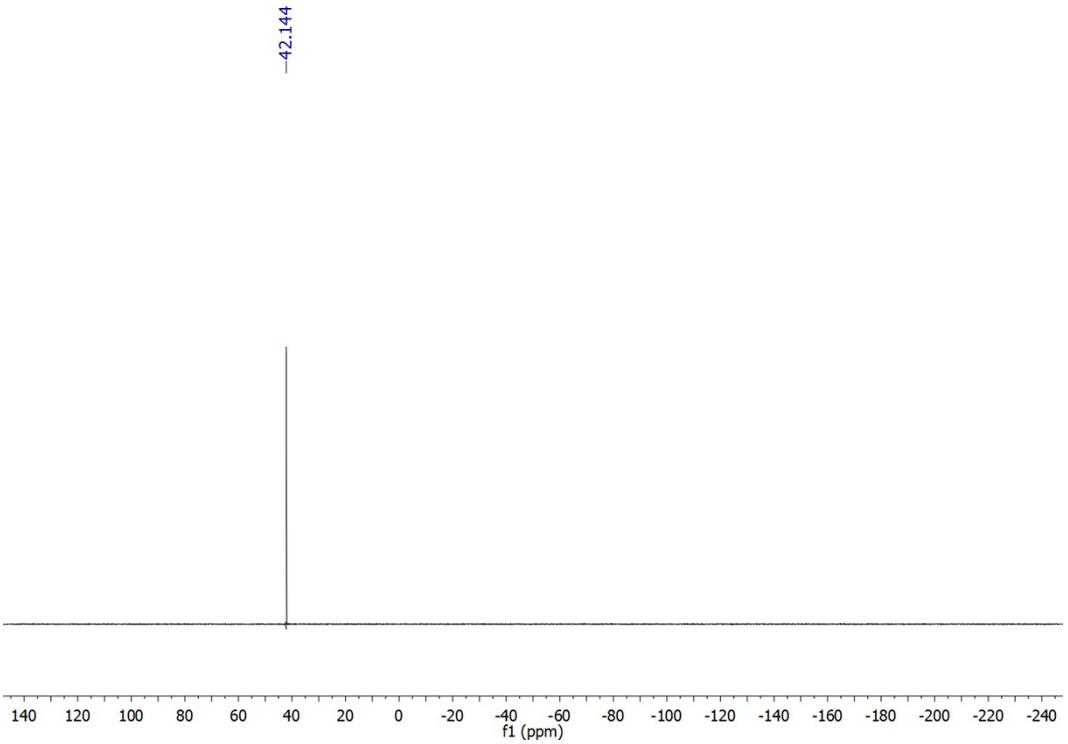

Figure S3. ^{31}P spectrum

Mass spectrometry was performed on a hybrid quadrupole time-of-flight (QTOF) analyzer, model QSTAR, Pulsar I, from AB Sciex (Framingham, MA, U.S.A.), as previously described.[S3] Samples were analyzed by direct infusion and ionized by electrospray ionization mass spectrometry (ESI–MS) with methanol as the mobile phase in positive reflector mode. High-resolution mass spectrometry (HR-MS) analysis (see Figure S4) was carried out by flow injection analysis combined with electrospray ionization mass spectrometry (FIA–ESI–MS) on a QTOF Agilent G6530A accurate mass QTOF liquid chromatography–mass spectrometry (LCMS) system (Agilent Technologies, Santa Clara, CA, U.S.A.). The sample was directly infused and ionized by ESI in negative reflector mode. Ionization was enhanced by JetStream technology, and the mobile phase was 99.9:0.1 (v/v) H_2O/formic acid. Data were processed with Masshunter Data Acquisition B.05.01 and Masshunter Qualitative Analysis B.07.00 software (Agilent Technologies). The analysis was performed at the *Servicio Interdepartamental de Investigación* (SIDI) from the Autonomous University of Madrid.

HR-MS m/z calcd. for $C_{16}H_{19}N_2O_3P$ $[M+H]^+$: 319.1211, found: 319.1205.

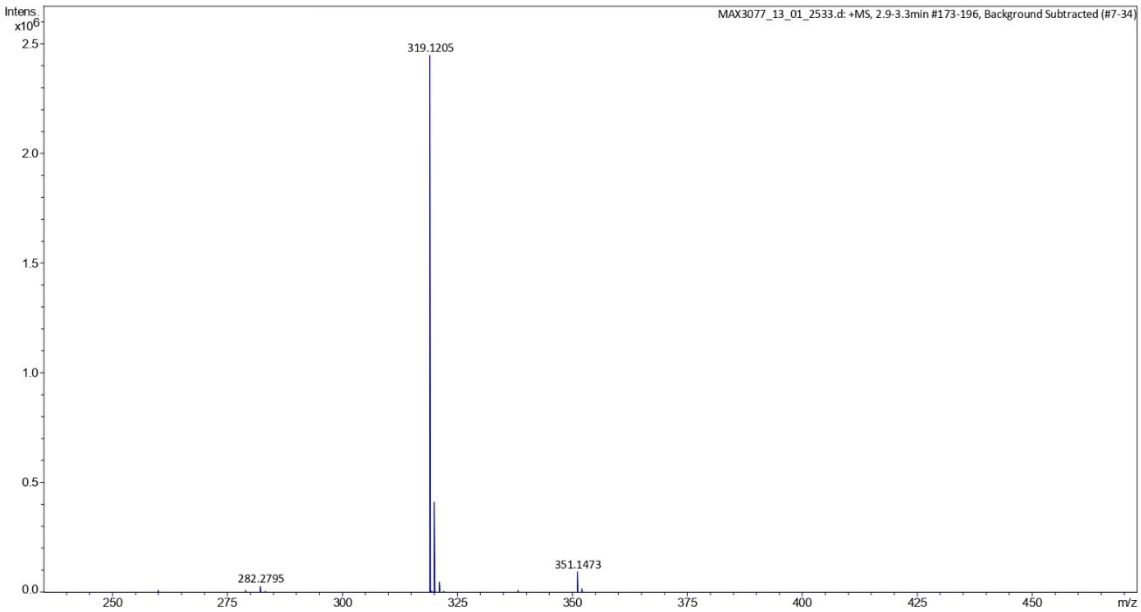

Figure S4. HR-MS spectrum

Electrochemical measurements. The electrochemical experiments were run, following conditions previously reported,[S3] at 22±1 ºC in a 3-electrode electrochemical cell configuration. Rotating gold disc electrodes (Pine, 5mm diameter) were used as working

electrodes. The reference electrode selected was BAS Ag/AgCl 3M (+210 mV vs SHE) and the counter electrode was a platinum wire. All redox potentials shown in the work are given vs. Ag/AgCl. The electrochemical experiments were recorded with an Autolab PGSTAT30 controlled with NOVA2 software (EcoChemie, NL). The electrochemical impedance spectroscopy was performed using the same cell configuration in presence 1 mM of the external redox probe $[Fe(CN)_6]^{3-/4-}$ (Merck Life Science S.L.U., Madrid, Spain) at a scanrate of 20 mV s^{-1}. The EIS measurements were carried out at a for-mal potential of the redox couple at which potential pulses of frequency between 0.1 Hz and 10 kHz were applied.

Before electrochemical measurements, gold electrode surface cleaning and modification was performed as previously described.[S3] Briefly, gold electrodes were immersed in "piranha" solution (3 H_2SO_4 98% (Merck Life Science S.L.U., Madrid, Spain): 1 H_2O_2 30% (Panreac, Madrid, Spain)) during 10 min. Afterwards the electrodes were rinsed with water and polished successively with alumina suspensions of 1, 0.3, and 0.05 µm in diameter, respectively during a total of 3 min. (*CAUTION: Piranha solution is especially dangerous, is corrosive, and may explode if contained in a closed vessel; it should be handled with special care.*) After rinsing, the electrodes were taken into an EtOH/H_2O 2:1 solution and immersed into an ultrasound bath during 15 min. Later the electrodes were taken into an electrochemical cell containing 0.5 NaOH and 20 electrochemical reductive cyclic voltammograms from 0 to -1.5 V using 200 mV·s^{-1} scan rate were performed to clean the gold surface. Final activation/cleaning step consisted on 25 oxidative cyclic voltammograms from 0 to +1.5 V using 100 mV·s^{-1} scan rate and H_2SO_4 0.1 M as electrolyte. Modification of gold electrodes was performed by immersion of the freshly cleaned electrodes into an ethanol solution containing 1 mM of 3-mercaptopropionic acid (MPA) (99%; Merck Life Science S.L.U., Madrid, Spain). Solutions were let to react overnight and clean by immersion in ethanol during 15 min. EH_3 was immobilized by physical adsorption by depositing on the electrode 10 µL of a solution containing the enzyme, 10 mg·mL^{-1} in 50 mM K_2HPO_4 buffer pH 6.5, during 1 hour. Then, the electrode was washed with water, afterwards it was taken into the electrochemical cell to determine the electrochemical response. Biomimetic EH_3, herein referred to as bEH_3, was prepared by incubating, at 30ºC, a solution containing the enzyme, 10 mg mL^{-1} (or ca. 0.26 mM) in 50 mM K_2HPO_4 buffer pH 6.5, with 0.63 mM of **2** (from a 126 mM stock solution in dimethyl sulfoxide), and after 10 min incubation biomimetic was extensively dialysed against 50 mM K_2HPO_4 buffer pH 6.5 using Pur-A-LyzerTM Maxi 1200 dialysis kit (Merck Life Science S.L.U., Madrid, Spain), overnight at 4 °C. The dialyzed biomimetic solution was recovered and incubated with 0.63 mM $Cu(NO_3)_2$ (from a 20 mM stock solution in the same buffer) for additional 10 minutes, after which the solution was again dialyzed to remove the unbound cation. The dialyzed protein solution was recovered and concentrated by ultra-filtration through low-adsorption hydrophilic 10000 nominal molecular weight limit cutoff membranes (regenerated cellulose, Amicon) to reach a final protein concentration of 0.26 mM (per monomer), and used inmediately.

Crystallization and X-ray structure determination of EH_3 complexed with 2. The complex bEH_3, was obtained by cocrystallization assays, using the sitting drop vapor diffusion method at 18°C. Initial crystallization conditions were explored by high-throughput techniques with a NanoDrop robot (Innovadyne Technologies, USA), incubating EH_3 (24 mg mL^{-1} in 40 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7, 100 mM NaCl) with inhibitor **2** (in 100% dimethylsulfoxide (DMSO)) to a final concentration of 10 mM inhibitor, for 2-3 hours before setting the drops. The commercial screens Index (Hampton Research, USA) and PACT++ (Jena Bioscience, Germany) were explored by mixing 250 nL protein solution with 125 nL of the reservoir. Further optimization led to suitable plaque-shaped crystals growing after four days, from 1 µL of the protein mixture and 0.5 µL of the reservoir containing 27% PEG3350, 0.1M Bis-Tris pH 6.5. For data collection, crystals were transferred to cryoprotectant solutions consisting of 29% PEG3350, 0.1M Bis-Tris pH 6.5 and 25% glycerol, before being cryocooled in liquid nitrogen. Diffraction data were collected using synchrotron radiation on the XALOC beamline at ALBA (Cerdanyola del Vallés, Spain). Diffraction images were processed with XDS[S4] and merged using AIMLESS from the CCP4 package.[S5] The crystals were indexed in the $P2_1$ space group, with four molecules in the asymmetric unit and 48% solvent content within the unit cell. The data-collection statistics are given in Table S2. The structure of bEH_3 was solved by Molecular Replacement with MOLREP,[S6] using the coordinates from the unliganded EH_3 as template (PDB code 6SXP). Crystallographic refinement was performed using the program REFMAC[S7] within the CCP4 suite with automatic local non-crystallographic symmetry (NCS) and amplitude-based twin refinement. Free R-factor was calculated using a subset of 5% randomly selected structure-factor amplitudes that were excluded from automated refinement. At the later stages, ligands were manually built into the electron density maps with COOT8[S8] and water molecules were included in the model, which, combined with more rounds of restrained refinement, reached the R factors listed in Table S2. For inhibitor **2**, not present in the Protein Data Bank, a model was built using MacPyMOLX11Hybrid (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). The model was used to automatically generate coordinates and molecular topologies with eLBOW suitable for REFMAC refinement.[S9] The figures were generated with PyMOL. The crystallographic statistics of bEH_3 are listed in Table S2.

Table S2. Crystallographic statistics of bEH_3

Crystal data	bEH_3
Space group	$P2_1$
Unit cell parameters	
a (Å)	56.76
b (Å)	189.39
c (Å)	75.95
β (º)	111.93
Data collection	
Beamline	XALOC (ALBA)
Wavelength (Å)	0.979181
Resolution (Å)	47.35-2.40 (2.47-2.40)
Data processing	
Total reflections	330,565 (26,293)
Unique reflections	57,906 (4,513)
Multiplicity	5.7 (5.8)
Completeness (%)	100.0 (100.0)
Mean *I*/σ (*I*)	8.1 (3.4)

R_{merge}[a] (%)	12.6 (51.9)
R_{pim}[b] (%)	5.7 (23.1)
Molecules per ASU	4
Refinement	
R_{work} / R_{free}[c] (%)	24.4/25.3
Nº of atoms/average B (Å^2)	10,429/45.41
Macromolecule	10,266/45.62
Ligands	39/45.32
Solvent	124/28.58
Ramachandran plot (%)	
Favoured	87.8
Outliers	0.9
RMS deviations	
Bonds (Å)	0.005
Angles (°)	1.440
PDB accession code	8PC7

[a] Rmerge = ∑hkl ∑i | Ii(hkl) – [I(hkl)]| / ∑hkl ∑i Ii(hkl), where Ii(hkl) is the ith measurement of reflection hkl and [I(hkl)] is the weighted mean of all measurements.

[b] Rpim = ∑hkl [1/(N - 1)] 1/2 ∑i | Ii(hkl) – [I(hkl)]| / ∑hkl ∑i Ii(hkl), where N is the redundancy for the hkl reflection.

[c] Rwork / Rfree = ∑hkl | Fo – Fc | / ∑hkl | Fo |, where Fc is the calculated and Fo is the observed structure factor amplitude of reflection hkl for the working / free (5%) set, respectively.

Preparation of biomimetic catalysts. Biomimetics, herein referred to as bEH_3 and bEH_{1AB1}, were prepared by incubating at 30ºC a solution containing the enzyme, 0.26 mM, in 50 mM K_2HPO_4 buffer pH 6.5, with 0.63 mM of **2** (from a 126 mM stock solution in DMSO). The reaction was monitored indirectly by measuring the biocatalytic activity using a pH indicator assay with glyceryl tripropionate as substrates, as described previously.[S10] In all cases, full inhibition was observed after 10 min, after which biomimetics were extensively dialysed against 50 mM K_2HPO_4 buffer pH 6.5 using Pur-A-LyzerTM Maxi 1200 dialysis kit (Merck Life Science S.L.U., Madrid, Spain), overnight at 4°C. The dialyzed biomimetic solutions were recovered and incubated with 0.63 mM $Cu(NO_3)_2$ (from a 20 mM stock solution in the same buffer) for additional 10 minutes, after which the solution was again dialyzed to remove the unbound cation. The dialyzed protein solution was recovered and concentrated by ultra-filtration through low-adsorption hydrophilic 10000 nominal molecular weight limit cutoff membranes (regenerated cellulose, Amicon) to reach a final protein concentration of 0.26 mM (per monomer), and used inmediately for activity tests.

Determination of hydrolytic activity. Before bionjugation at large, esterase activity inhibition was measured over time by following the specific activity (units/mg protein) using glyceryl tripropionate (Merck Life Science S.L.U., Madrid, Spain) using the Phenol Red pH indicator assay.[S1] The activity was calculated by determining the absorbance per minute from the slopes generated, and by applying the following formula (Equation 1):

$$Rate\left(\frac{\mu mol}{\min mg\ protein}\right) = \frac{\frac{\Delta Abs}{min}}{8450\ M-1cm-1} * \frac{1}{0.4\ cm} * \frac{10^6\ \mu M}{1\ M} * 0.000044\ L * \frac{1}{mg\ protein}$$

Assuming the following parameters:

- Extinction coefficient (ε) phenol red at 550 nm and pH 8.0: 8450 $M^{-1}cm^{-1}$
- Total assay volume: 44 µL
- Total amount protein: 0.012 mM

The reaction conditions were set up in 384 well plate, as follows.

- 40 µL 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS) buffer pH 8.0 with 0.45 mM Phenol Red are added to each well.
- Then, 2 µL enzyme solution (from a 0.26 mM stock solution) are added (final enzyme concentration 0.012 mM).
- Then, 1 µL of inhibitor **2** stock solution (1.26 mM stock solution in DMSO) are added (final inhibitor **2** concentration 0.03 mM).
- After incubation for a desired time, 1 µL of glyceryl tripropionate stock solution (100 mg/ml in acetonitrile) are added.
- Measure the decrease of absorbance at 550 nm in a spectrophotometer at 30 ˚C every one min.

All values, in triplicate, were corrected for nonenzymatic transformation. The absence of activity was defined as at least a twofold background signal as described.[S1]

Catecholase activity determination and High Performance Liquid Chromatography (HPLC) analysis. Catecholase activity was evaluated (at 30ºC) by following the conversion of 3,5-di-tert-butylcatechol to 3,5-di-tert-butyl-O-benzoquinone. Briefly, 2 µL of 3,5-di-tert-butylcatechol (from a stock solution of 225 mM in dimethyl sulfoxide) were added to 88 µL of 50 mM K_2HPO_4 buffer at pH 6.5. Then, 10 µL of biomimetic enzyme solution (from a stock solution of 2.6 mM in 40 mM HEPES buffer, pH 7.0) were added. After 60 min, reactions (in triplicates) were stopped by adding 900 µL HPLC-grade methanol and the reaction products analysed by HPLC, compared to a control reaction without enzyme. For the HPLC analysis, the samples were filtered employing a 0.45 µm nylon filter and the presence of the compounds were quantified by HPLC analysis, performed using a quaternary pump (model 600, Waters) coupled to

an autosampler (Varian ProStar, model 420). The injection volume was 10 µL. The column was a Zorbax Eclipse Plus C-18 (4.6 x 100 mm, 3.5 µm, Agilent Technologies) with a constant temperature of 40 °C. The mobile phase consisted of an isocratic mixture of acetonitrile/H_2O 58:42 (v/v) acidified with a 0.1% (v/v) of formic acid and degassed with helium. The flow rate was 0.8 mg mL^{-1} during 14 min of analysis. The detection of peaks was carried out using a photodiode array detector (ProStar, Varian). Quantification was performed at 275 nm and integration was carried out using the software Varian Star LC workstation 6.41. The compound concentration of each sample was quantified from the peak area extracted from the chromatograms. Calibration curves for 3,5-di-*tert*-butylcatechol and 3,5-di-*tert*-butyl-O-benzoquinone (both provided by Merck Life Science S.L.U., Madrid, Spain), between 0 and 5 mM, were performed to extract exact concentrations in reaction mixtures.

Oxidation of catechin: spectrophotometer and HPLC. The reaction conditions for the oxidation of (+)-catechin or (−)-catechin (Merck Life Science S.L.U., Madrid, Spain) were set up in 96 well plate, as follows.

- 200 µL 50 mM K_2HPO_4 buffer at pH of 6.5 are added to each well.
- 50 µL of (+) or (−)-catechin solution (10.4 mg mL^{-1} stock solution in acetonitrile) are added (final concentration, 2 mg mL^{-1}, or 6.9 mM).
- Then, 10 µL biomimetic solution (from a 0.26 mM stock solution, or 10 mg mL^{-1}) are added (final concentration, 0.01 mM or 0.38 mg mL^{-1}).
- Measure the decrease of absorbance at 290 nm (catechin) and increase of absorbance at 330 nm (catechin quinone) in a spectrophotometer at 30 ˚C every one min. All values, in triplicate, were corrected for nonenzymatic transformation.

Specific activity (unit/mg protein) is calculated by determining the absorbance per minute from the slopes generated, and by applying the following formula (Equation 2):

$$Rate\left(\frac{\mu mol}{\min \mu mol\ protein}\right) = \frac{\frac{\Delta Abs}{min}}{10230\ M-1cm-1} * \frac{1}{1\ cm} * \frac{10^6\ \mu M}{1\ M} * 0.00026\ L * \frac{1}{\mu mol\ protein}$$

Assuming the following parameters:

- Extinction coefficient (ε) catechin: 10230 $M^{-1}cm^{-1}$
- Total assay volume: 260 µL
- Total amount protein: 0.0026 µmol or 0.1 mg

For HPLC analysis, the samples were diluted 1 : 4 in methanol and filtered employing a 0.45 µm nylon filter and the presence of the compounds were analyzed by HPLC, performed using a quaternary pump (model 600, Waters) coupled to an autosampler (Varian ProStar, model 420). The injection volume was 5 µL. The column was a Zorbax Eclipse Plus C-18 (4.6 x 100 mm, 3.5 µm, Agilent Technologies) with a constant temperature of 40 °C. The mobile phase consisted of a gradient with acetonitrile and water, both acidified with a 0.1% (v/v) of formic acid and degassed with helium. The mobile phase was fixed at 5 % of acetonitrile during 1 minute and, after that, a gradient from 5 % to 50 % of acetonitrile in 3 minutes was carried out, keeping the acetonitrile fixed at 50 % during 3 minutes more. After that, the mobile phase return to initial conditions in 3 minutes and the column was equilibrated during 10 minutes before the next injection. The flow rate was 0.8 mL min^{-1} during the 20 minutes of analysis. The detection of peaks was carried out using a photodiode array detector at 280 and 350 nm (ProStar, Varian). Relative amount of catechin (see Figure S5) was obtained from the peak area at 280 nm (see Figure S6) employing the software Varian Star LC workstation 6.41. We considered 100 % the area of the total amount of catechin added.

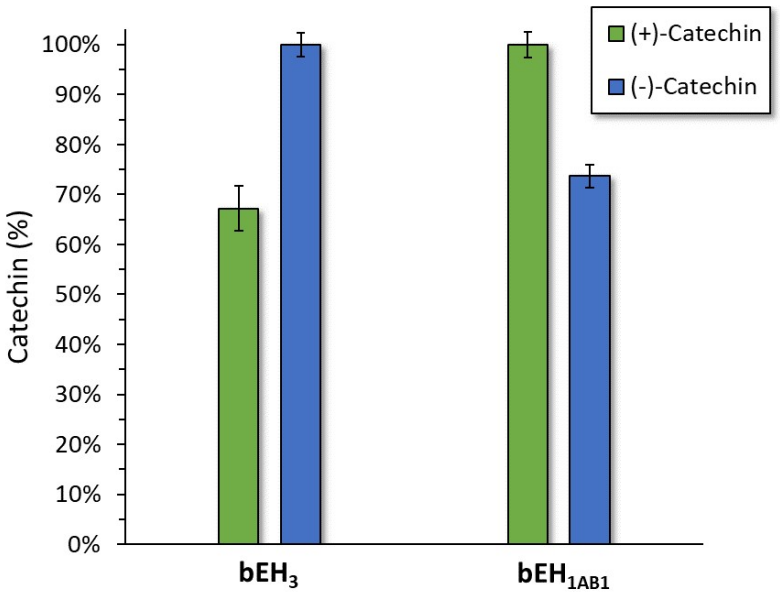

Figure S5. Conversion of (+/−)-catechin to (+/−)-catechin quinone by bEH_3 and bEH_{1AB1}. Reaction conditions: [biomimetic, bEH_3 or bEH_{1AB1}]: 0.01 mM; [(+/−)-catechin]: 6.9 mM; Temperature: 30ºC; Incubation time: 1 h; total volume: 100 µL 50 mM K_2HPO_4 buffer pH 6.5.

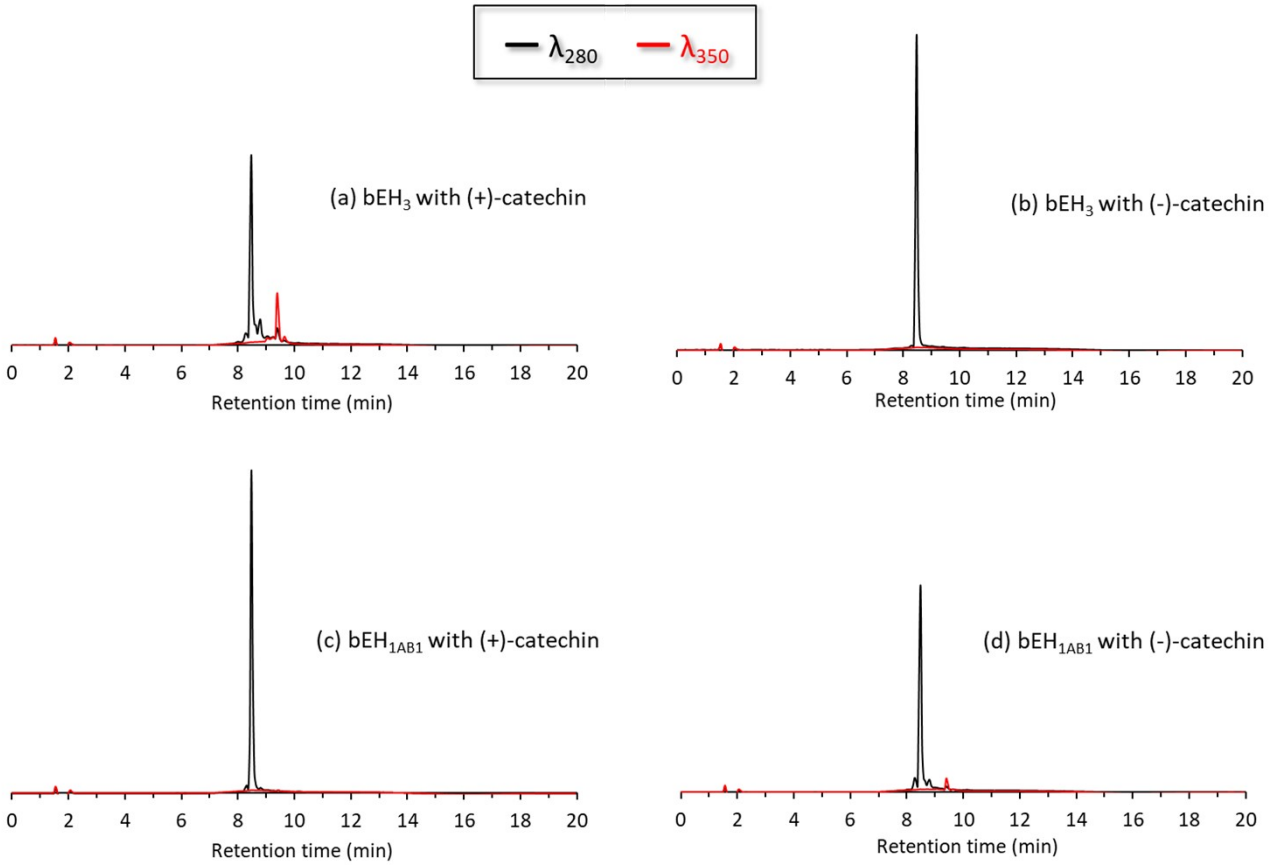

Figure S6. Representative HPLC chromatograms of the reaction products when (+)-catechin or (−)-catechin was treated with bEH_{1A1} and bEH_3 during 30 min. Reaction conditions: [biomimetic, bEH_3 or bEH_{1AB1}]: 0.01 mM; [(+)-catechin or (−)-catechin]: 6.9 mM; Temperature: 30ºC; Incubation time: 1 h; total volume: 100 µL 50 mM K_2HPO_4 buffer at pH of 6.5.

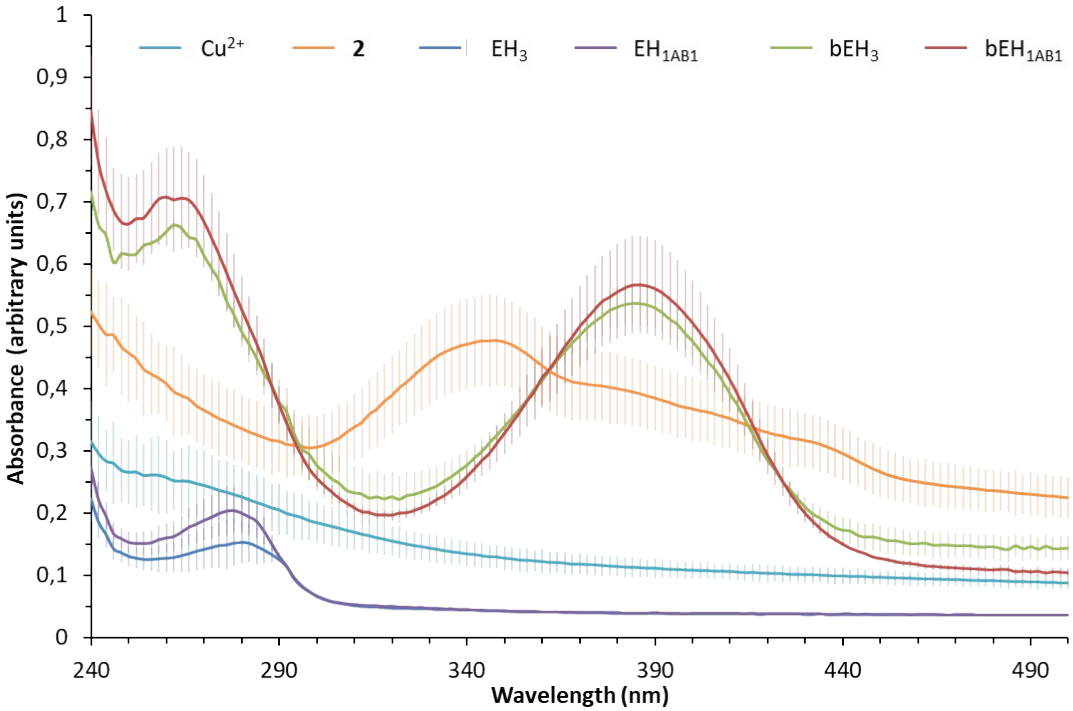

Figure S7. UV/Vis spectra of EH_3 and EH_{1AB1} and the corresponding biomimetics bEH_3 and bEH_{1AB1}. The spectra were recorded in a Synergy HT Multi-Mode Microplate Reader (Biotek Instruments, Winooski, VT, USA) at wavelength 240 to 500 nm in 96-well plates (ref. 655801, Greiner Bio-One GmbH, Kremsmünster, Austria) using the enzymes (15 µM) prepared with an excess (120 µM) of **2** and Cu^{2+}, in 200 µl of 50 mM K_2HPO_4 buffer pH 6.5, room temperature (25ºC) and 10 min incubations. Spectra of Cu^{2+}, **2** and unmodified enzymes, used at the same concentrations as for the formation of biomimetics, are shown as controls. Shown are average values with the standard deviation of measurements in triplicate. Figure made using Excel 2019. λ_{max} values were obtained with Gen5 2.00 software (Biotek Instruments, Winooski, VT, USA): λ_{max} for EH_3, 280 nm; λ_{max} for EH_{1AB1}, 278 nm; λ_{max} for **2**, 348 nm; λ_{max} for bEH_3, 384 nm; λ_{max} for bEH_{1AB1}, 386 nm.

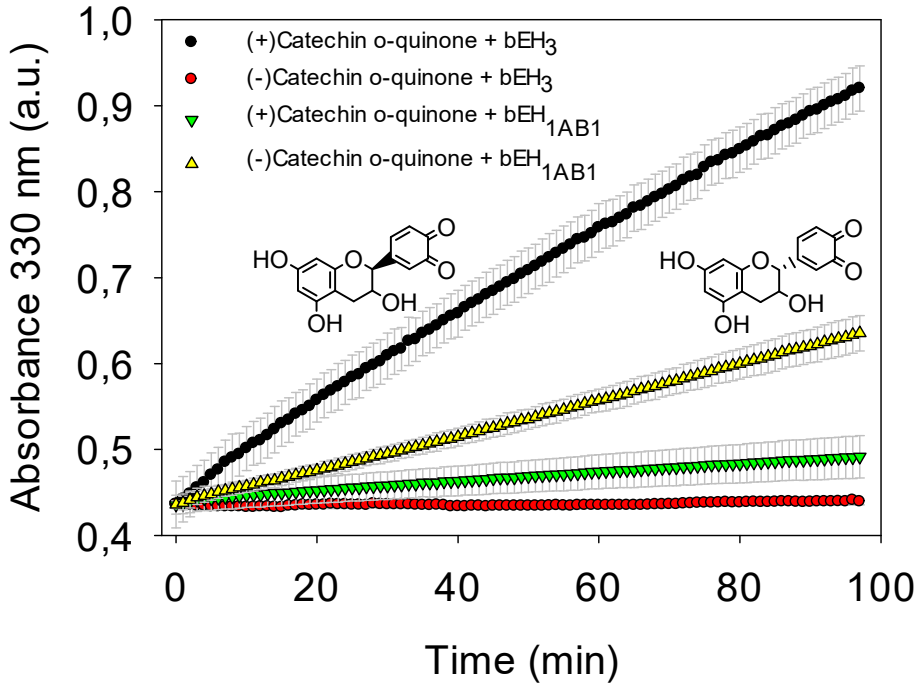

Figure S8. Catechin oxidation by bEH_{1A1} and bEH_3. Reaction conditions: [biomimetic, bEH_3 or bEH_{1AB1}]: 0.01 mM; [(+)-catechin or (–)-catechin]: 6.9 mM; temperature: 30 °C; incubation time: 100 min; total volume: 250 µL of a 50 mM K_2HPO_4 buffer with a pH 6.5. (+/–) Catechin o-quinone formation was followed spectrophotometrically at 330 nm. According to Equation 2, the reaction rates were as follows: bEH_3 and (+)-catechin: 1.191 ± 0.010 min^{-1}; bEH_3 and (–)-catechin: 0.013 ± 0.001 min^{-1}; bEH_{1AB1} and (+)-catechin: 0.128 ± 0.001 min^{-1}; bEH_{1AB1} and (–)-catechin: 0.496 ± 0.001 min^{-1}.

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Author Contributions

L.F.-L. and I.C.-R. contributed equally to this work. J.S.-A. and M.F., lead this contribution. M.F. wrote the original manuscript, which was further written through contributions from M.F., J.S.-A., M.P., and P.S. All the authors have given approval to the final version of the manuscript. I.C.-R. and J.S.-A. performed the crystallization and X-ray structure determinations. J. A.M. and M.P. contributed together with L.F.-L. to the electrochemical measurements and discussion. L.F.-L., C.C., and M.F contributed to protein expression, purification and characterization. J.L.G.-A. and F.J.P. performed HPLC analysis of the reaction products. A.K.R and J.M contributed to inhibitor **2** synthesis and characterisation. M.F. and P.S. conceived the strategy.