Supporting Information

Chemical Optimization of Whole-Cell Transfer Hydrogenation Using Carbonic Anhydrase as Host Protein

Johannes G. Rebelein[+], Yoann Cotelle[+], Brett Garabedian and Thomas R. Ward*

Department of Chemistry, University of Basel, Mattenstrasse 24a, BPR 1096, 4058 Basel, Switzerland

(+ authors contributed equally, * corresponding author: thomas.ward@unibas.ch)

1. Supporting Methods 2
   a. General aspects 2
   b. Cloning of constructs for periplasmic compartmentalization and surface display of CAII. ..2
   c. Culture and expression conditions. 2
   d. Synthesis 3
   e. Determination of the cofactor's **6**-**10** dissociation constant K_d. 10
   f. Transfer hydrogenation assays and ICP-MS sample preparation 11
   g. Labelling of CAII expressing cells with fluorescent probe **4** 12
   h. Labelling of CAII expressing cells with anti-CAII antibodies 12
   i. Microscopy analysis of probe **4** and antibody-labelled *E. coli* cells 13
   j. Flow-cytometry analysis of probe **4** and antibody-labelled *E. coli* cells 13
   k. Crystal structure solution and analysis of CAII soaked with cofactor **7**-**10** 13
2. Supporting Tables 15
3. Supporting Figures 23
4. References 39

1. Supporting Methods

a. General aspects

All commercially available chemicals were purchased from Sigma-Aldrich, ABCR, TCI Europe, Acros Organics, Alfa Aesar, Fluka, Fluorochem, Merck or Ukrorgsyntez Ltd. and used without further purification. ^{1}H and ^{13}C spectra were recorded on a Bruker 500 MHz. Chemical shifts are reported in ppm (parts per million). Signals are quoted as s (singlet), d (doublet), t (triplet), bs (broad singlet) and m (multiplet). Electron-Spray Ionization Mass Spectra (ESI-MS) were recorded on a Bruker FTMS 4.7T bioAPEX II. High-resolution mass spectra (HRMS) were measured on a Bruker maXis 4G QTOF ESI mass spectrometer.

b. Cloning of constructs for periplasmic compartmentalization and surface display of CAII.

The CAII gene was amplified by polymerase chain reaction (PCR) from the plasmid "CAII^{cytoplasm}" (Table S1) with the primers NheI-CAII_fwd and SacI-CAII_rev (Table S2) to introduce the restrictions sites NheI and SacI. The PCR product and the target vectors pET-30b_Sav^{peri}[1] and pET-30b_Lpp-OmpA-Sav$_{WT}$[2] (Table S1) were digested with the restriction enzymes NheI and SacI. To digest the streptavidin (Sav) gene, AgeI was added to the vector digest. The vectors were dephosphorylated with antarctic phosphatase and gel-purified. The CAII-insert was ligated with T4 DNA ligase into the cut vectors resulting in the plasmids "CAII^{periplasm}" (DNA sequence collected in Table S3) and "CAII$^{surface\ display}$" (DNA sequence collected in Table S4). For the enzyme digest, dephosphorylation and ligation NEB protocols were followed.

c. Culture and expression conditions.

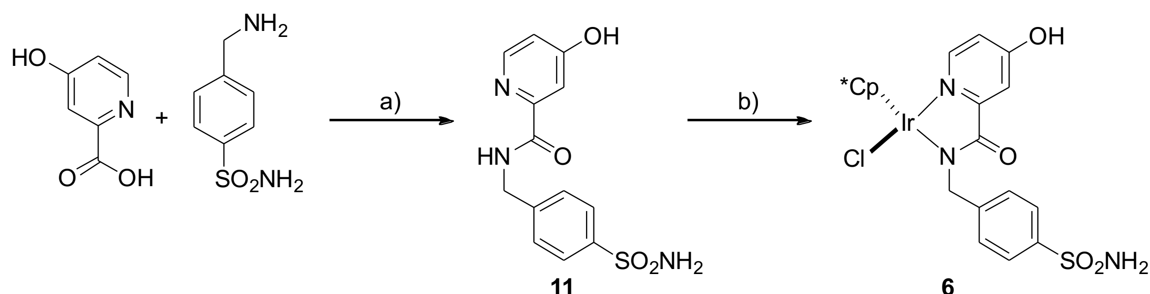
For this study, we used the strain *Escherichia coli* TOP10(DE3), previously reported.[1] The culture and expression conditions were adapted from previous publications.[1-3] In short, TOP10(DE3) *E. coli* cells containing an empty vector or plasmids for the compartmentalization of CAII in the cytoplasm, periplasm or on the cell surface (Table S1) were cultured independently as triplicate. Overnight cultures in Luria–Bertani (LB) medium, supplemented with the appropriate antibiotic,

were used to inoculate the main culture (50 mL modified ZYM-5052 rich medium, see Table S5) in baffled Erlenmeyer flasks (volume: 250 mL) with an OD_{600} = 0.05. The flasks were incubated shaking at 220 rpm and 37 °C. At an OD_{600} between 0.5 and 1, the cultures were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG, 250 µM final concentration) and kept shaking at 220 rpm and 30 °C for another 4 h.

d. Synthesis

Cofactor 5 was synthesized according to a published procedure.[4]

Profluorescent substrate 1 was synthesized according to a published procedure.[3]

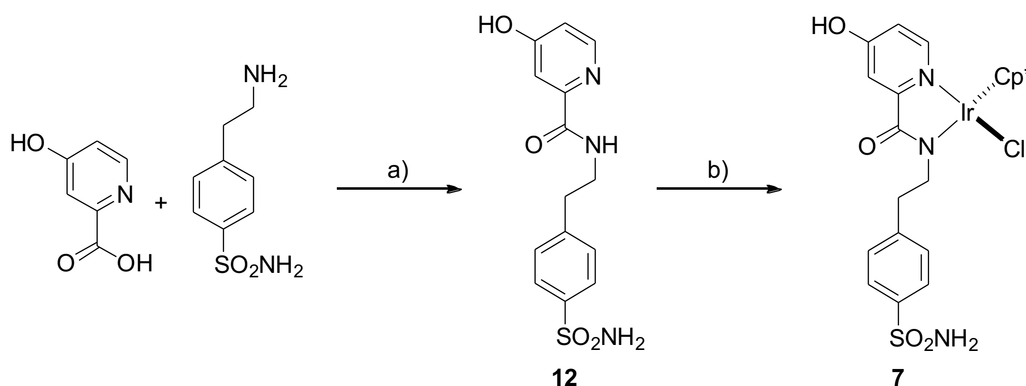


Scheme S1: a) DCC, HOBt, EDCI·HCl, DIPEA, DMF, 70 °C, 18 h, 63% b) $[IrCp^*Cl_2]_2$, EtOH, 80 °C, 2 h, 34%.

Ligand 11. 4-hydroxypicolinic acid (50 mg, 0.36 mmol), HOBt (100 mg, 0.65 mmol), EDCI·HCl (140 mg, 0.73 mmol) and DIPEA (0.125 µL, 0.76 mmol) were added to a solution of 4-(aminomethyl)benzenesulfonamide (124 mg, 0.67 mmol) in DMF (3 mL). The solution was heated at 70 °C for 18 h. The solvents were removed *in vacuo*. The crude was purified using silica gel column chromatography (CH_2Cl_2/MeOH 95/5), followed by trituration in DCM to give ligand **11** (70 mg, 63%) as a white solid. R_f (CH_2Cl_2/MeOH 90/10): 0.24; ^{1}H NMR (500 MHz, DMSO-d_6): 11.05 (bs, 1H), 9.37 (t, $^3J_{H-H}$ = 6.5 Hz, NH, 1H), 8.31 (bs, 1H), 7.76 (d, $^3J_{H-H}$ = 8.0 Hz, 2H), 7.47 (d, $^3J_{H-H}$ = 8.0 Hz, 2H), 7.42 (bs, 1H), 7.30 (s, 2H), 6.92 (bs, 1H), 4.52 (d, $^3J_{H-H}$ = 6.5 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6): 165.2, 164.0, 151.6, 149.6, 143.6, 142.6, 127.6, 125.7, 113.9, 109.7, 42.1; MS

(ESI, +ve): 308 (100, $[M+H]^+$); HRMS (ESI, +ve) calcd for $C_{13}H_{14}N_3O_4S$ ($[M+H]^+$): 308.0700, found: 308.0695.

Cofactor 6. The $[IrCp^*Cl_2]_2$ precursor (20 mg, 0.03 mmol) was added under N_2 to a solution of ligand **11** (20 mg, 0.06 mmol) in EtOH (5 mL). The solution was heated at 80 °C for 2 h. The solvents were removed *in vacuo*. The remaining solid was triturated in DCM (5 mL) three times. Purification of the residue using silica gel column chromatography (CH_2Cl_2/MeOH 94/6) afforded cofactor **6** (14 mg, 34%) as a yellow solid. R_f (CH_2Cl_2/MeOH 90/10): 0.16; ^1H NMR (500 MHz, DMSO-d_6): 11.76 (bs, 1H), 8.39 (d, $^3J_{H\text{-}H}$ = 6.4 Hz, 1H), 7.67 (d, $^3J_{H\text{-}H}$ = 7.9 Hz, 2H), 7.48 (d, $^3J_{H\text{-}H}$ = 7.9 Hz, 2H), 7.26 (s, 2H), 7.18 (bs, 1H), 7.00 (bs, 1H), 4.83 (bs, 2H), 1.52 (s, 15 H); ^{13}C NMR (126 MHz, DMSO-d_6): 171.5, 166.6, 155.8, 152.0, 146.0, 142.0, 128.0, 125.3, 115.6, 112.3, 86.2, 55.4, 54.2, 9.1; MS (ESI, +ve): 634 (100, $[M\text{-}Cl]^+$); HRMS (ESI, +ve) calcd for $C_{23}H_{27}IrN_3O_4S$ ($[M\text{-}Cl]^+$): 634.1345, found: 634.1348.

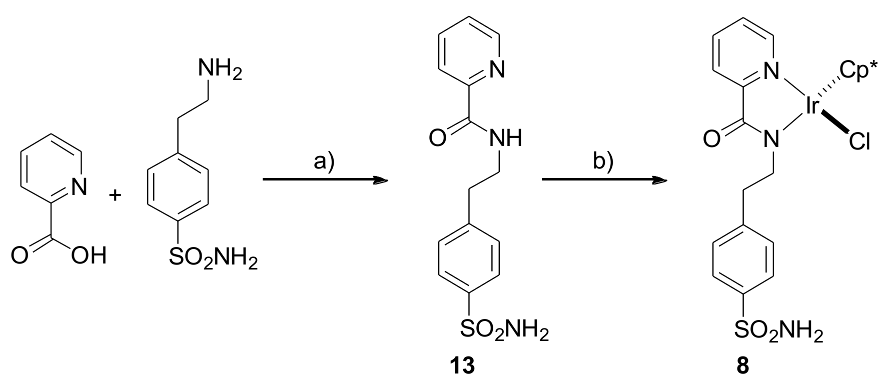


Scheme S2: a) DCC, HOBt, EDCI·HCl, DIPEA, DMF, 70 °C, 18 h, 95% b) $[IrCp^*Cl_2]_2$, EtOH, 80 °C, 2 h, 39%.

Ligand 12. 4-hydroxypicolinic acid (50 mg, 0.36 mmol), HOBt (100 mg, 0.74 mmol), EDCI·HCl (140 mg, 0.73 mmol) and DIPEA (125 µL, 0.76 mmol) were added to a solution of 4-(aminoethyl)benzenesulfonamide (140 mg, 0.69 mmol) in DMF (3 mL). The solution was heated at 70 °C for 18 h. The solvents were removed *in vacuo*. The crude was purified using silica gel column chromatography (CH_2Cl_2/MeOH 92/8), followed by trituration in DCM to give ligand **12** (110 mg, 95%) as a white solid. R_f (CH_2Cl_2/MeOH 90/10): 0.21; ^1H NMR (500 MHz, DMSO-d_6): 10.98 (s, 1H), 8.76 (t, $^3J_{H\text{-}H}$ = 6.5 Hz, NH, 1H), 8.30 (d, $^3J_{H\text{-}H}$ = 5.5 Hz, 1H), 7.73 (d, $^3J_{H\text{-}H}$ = 8.0 Hz, 2H), 7.43-7.41

(m, 3H), 7.28 (s, 2H), 6.91 (dd, $^4J_{H\text{-}H}$ = 2.5 Hz, $^3J_{H\text{-}H}$ = 5.5 Hz, 1H), 3.54 (q, $^3J_{H\text{-}H}$ = 6.5 Hz, 2H), 2.93 (t, $^3J_{H\text{-}H}$ = 6.5 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6): 165.6, 164.2, 152.2, 150.2, 144.1, 142.5, 129.5, 126.2, 114.1, 109.9, 40.2, 35.3; MS (ESI, +ve): 322 (100, [M+H]$^+$); HRMS (ESI, +ve) calcd for $C_{14}H_{16}N_3O_4S$ ([M+H]$^+$): 322.0856, found: 322.0852.

Cofactor 7. The [IrCp*Cl$_2$]$_2$ precursor (25 mg, 0.03 mmol) was added under N_2 to a solution of ligand **12** (20 mg, 0.06 mmol) in EtOH (5 mL). The solution was heated at 80 °C for 2 h. The solvents were removed *in vacuo*. The remaining solid was triturated in DCM. Purification of the residue using silica gel column chromatography (CH_2Cl_2/MeOH 92/8) gave **7** (16 mg, 39%) as a yellow solid. R_f (CH_2Cl_2/MeOH 90/10): 0.11; ^{1}H NMR (500 MHz, DMSO-d_6): 11.79 (bs, 1H), 8.36 (d, $^3J_{H\text{-}H}$ = 5.3 Hz, 1H), 7.76 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 2H), 7.44 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 2H), 7.30 (s, 2H), 7.19 (d, $^3J_{H\text{-}H}$ = 2.4 Hz, 1H), 7.00 (dd, $^4J_{H\text{-}H}$ = 2.4 Hz, $^3J_{H\text{-}H}$ = 5.3 Hz, 1H), 4.46 (td, $^3J_{H\text{-}H}$ = 9.6 Hz, $^2J_{H\text{-}H}$ = 4.6 Hz, 1H), 3.24 (td, $^3J_{H\text{-}H}$ = 9.6 Hz, $^2J_{H\text{-}H}$ = 4.6 Hz, 1H), 2.99 (td, $^3J_{H\text{-}H}$ = 9.6 Hz, $^2J_{H\text{-}H}$ = 4.6 Hz, 1H), 2.77 (td, $^3J_{H\text{-}H}$ = 9.6 Hz, $^2J_{H\text{-}H}$ = 4.6 Hz, 1H), 1.63 (s, 15 H); ^{13}C NMR (151 MHz, DMSO-d_6): 169.8, 166.1, 155.9, 151.2, 145.1, 141.6, 128.9, 125.8, 115.0, 111.8, 94.6, 85.6, 50.5, 35.2, 8.6; MS (ESI, +ve): 648 (100, [M-Cl]$^+$); HRMS (ESI, +ve) calcd for $C_{24}H_{29}IrN_3O_4S$ ([M-Cl]$^+$): 648.1502, found: 648.1507.

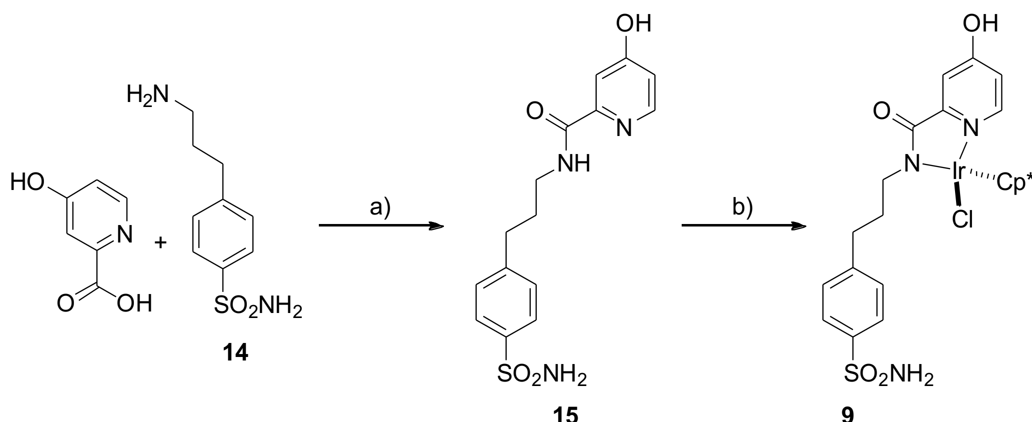


Scheme S3: a) DCC, HOBt, EDCI·HCl, DIPEA, DMF, 70 °C, 18 h, 47% b) [IrCp*Cl$_2$]$_2$, DIPEA, EtOH, 80 °C, 2 h, 29%.

Ligand 13. Picolinic acid (44 mg, 0.36 mmol), HOBt (50 mg, 0.37 mmol), EDCI·HCl (70 mg, 0.37 mmol) and DIPEA (125 µL, 0.76 mmol) were added to a solution of 4-(2-aminoethyl)benzenesulfonamide (70 mg, 0.34 mmol) in DMF (3 mL). The solution was heated at

70 °C for 18 h. The solvents were removed *in vacuo*. Purification of the residue using silica gel column chromatography (CH_2Cl_2/MeOH 98/2) gave **13** (52 mg, 47%) as a white solid. R_f (CH_2Cl_2/MeOH 90/10): 0.69; ^{1}H NMR (500 MHz, DMSO-d_6): 8.88 (t, $^3J_{H-H}$ = 6.2 Hz, 1H), 8.63-8.62 (m, 1H), 8.03-7.96 (m, 1H), 7.75-7.72 (m, 2H), 7.60-7.57 (m, 1H), 7.44-7.41 (m, 2H), 7.28 (s, 2H), 3,60-3.55 (m, 2H), 2,94 (t, $^3J_{H-H}$ = 7.3 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6): 163.8, 149.9, 148.4, 143.6, 142.0, 137.8, 129.1, 126.5, 125.7, 121.8, 40.4, 34.8; MS (ESI, +ve): 306 (100, $[M+H]^+$); HRMS (ESI, +ve) calcd for $C_{14}H_{16}N_3O_3S$ ($[M+H]^+$): 306.0907, found: 306.0910.

Cofactor 8. The $[IrCp^*Cl_2]_2$ precursor (20 mg, 0.025 mmol) was added under N_2 to a solution of ligand **13** (15 mg, 0.05 mmol) in EtOH (5 mL). DIPEA (9 µL) was added and the solution was heated at 80 °C for 2 h. The precipitate was filtered and washed with DCM and MeOH to give **8** (13 mg, 29%) as a yellow solid. R_f (CH_2Cl_2/MeOH 90/10): 0.30; ^{1}H NMR (500 MHz, DMSO-d_6): 8.70 (d, $^3J_{H-H}$ = 5.3 Hz, 1H), 8.08 (td, $^3J_{H-H}$ = 1.3 Hz, $^3J_{H-H}$ = 7.7 Hz 1H), 7.84 (dd, $^3J_{H-H}$ = 1.3 Hz, $^3J_{H-H}$ = 7.7 Hz, 1H), 7.76 (d, $^3J_{H-H}$ = 8.3 Hz, 2H), 7.66 (m, 1H), 7.45 (d, $^3J_{H-H}$ = 8.3 Hz, 2H), 7.28 (s, 2H), 4.54-4.46 (m, 1H), 3.31-3.23 (m, 1H), 3.04-2.97 (m, 1H), 2.83-2.76 (m, 1H), 1.64 (s, 15 H); ^{13}C NMR (126 MHz, DMSO-d_6): 169.7, 154.3, 150.4, 145.0, 141.7, 139.0, 128.8, 127.6, 125.8, 124.6, 86.2, 50.3, 35.1, 8.5; MS (ESI, +ve): 632 (100, $[M-Cl]^+$); HRMS (ESI, +ve) calcd for $C_{24}H_{29}IrN_3O_3S$ ($[M-Cl]^+$): 632.1553, found: 632.1547.

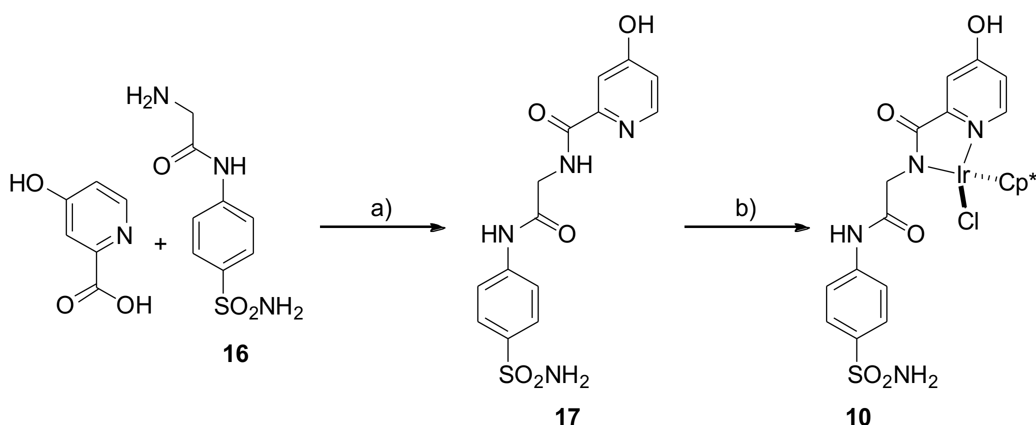


Scheme S4: a) DCC, HOBt, EDCI·HCl, DIPEA, DMF, 70 °C, 18 h, 29% b) $[IrCp^*Cl_2]_2$, EtOH, 80 °C, 2 h, 10%.

Compound 16 was synthesized according to a reported procedure.[5]

Ligand 15. 4-hydroxypicolinic acid (50 mg, 0.36 mmol), HOBt (100 mg, 0.74 mmol), EDCI·HCl (140 mg, 0.73 mmol) and DIPEA (125 µL, 0.76 mmol) were added to a solution of 4-(aminopropyl)benzenesulfonamide **14** (153 mg, 0.71 mmol) in DMF (3 mL). The solution was heated at 70 °C for 18 h. The solvents were removed *in vacuo*. The crude was purified using silica gel column chromatography (CH_2Cl_2/MeOH 93/7), then it was dissolved in MeOH and precipitated by addition of Et_2O to give ligand **15** (35 mg, 29%) as a white solid. R_f (CH_2Cl_2/MeOH 90/10): 0.22; ^{1}H NMR (500 MHz, DMSO-d_6): 11.07 (s, 1H), 8.77 (bs, NH, 1H), 8.31 (bs, 1H), 7.73 (d, $^3J_{H\text{-}H}$ = 6.3 Hz, 2H), 7.45-7.40 (m, 3H), 7.28 (s, 2H), 6.93 (bs, 1H), 3.31-3.26 (m, 2H), 2.67 (t, $^3J_{H\text{-}H}$ = 6.1 Hz, 2H), 1.84 (t, $^3J_{H\text{-}H}$ = 6.1 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6): 165.1, 163.9, 151.9, 149.7, 146.0, 141.7, 128.8, 125.7, 113.6, 109.5, 53.31, 32.4, 30.6, 18.0, 16.7.; MS (ESI, +ve): 336 (100, $[M+H]^+$).

Cofactor 9. The $[IrCp^*Cl_2]_2$ precursor (20 mg, 0.03 mmol) was added under N_2 to a solution of ligand **15** (25 mg, 0.06 mmol) in EtOH (5 mL). The solution was heated at 80 °C for 2 h. The solvents were removed *in vacuo*. The remaining solid was triturated in DCM. Purification of the residue using silica gel column chromatography (CH_2Cl_2/MeOH 94/6) gave **9** (4 mg, 10%) as a yellow solid. R_f (CH_2Cl_2/MeOH 90/10): 0.11; ^{1}H NMR (500 MHz, DMSO-d_6): 11.68 (bs, 1H), 8.32 (d, $^3J_{H\text{-}H}$ = 6.4 Hz, 1H), 7.70 (d, $^3J_{H\text{-}H}$ = 6.4 Hz, 2H), 7.41 (d, $^3J_{H\text{-}H}$ = 6.4 Hz, 2H), 7.23 (s, 2H), 7.15 (bs, 1H), 6.97 (bs, 1H), 4.40-4.31 (m, 1H), 3.10-3.01 (m, 1H), 2.75-2.68 (m, 1H), 2.58-2.62 (m, 1H), 1.83-1.76 (m, 2H), 1.56 (s, 15 H); MS (ESI, +ve): 662 (100, $[M-Cl]^+$); HRMS (ESI, +ve) calcd for $C_{25}H_{31}IrN_3O_4S$ ($[M-Cl]^+$): 662.1658, found: 662.1671.



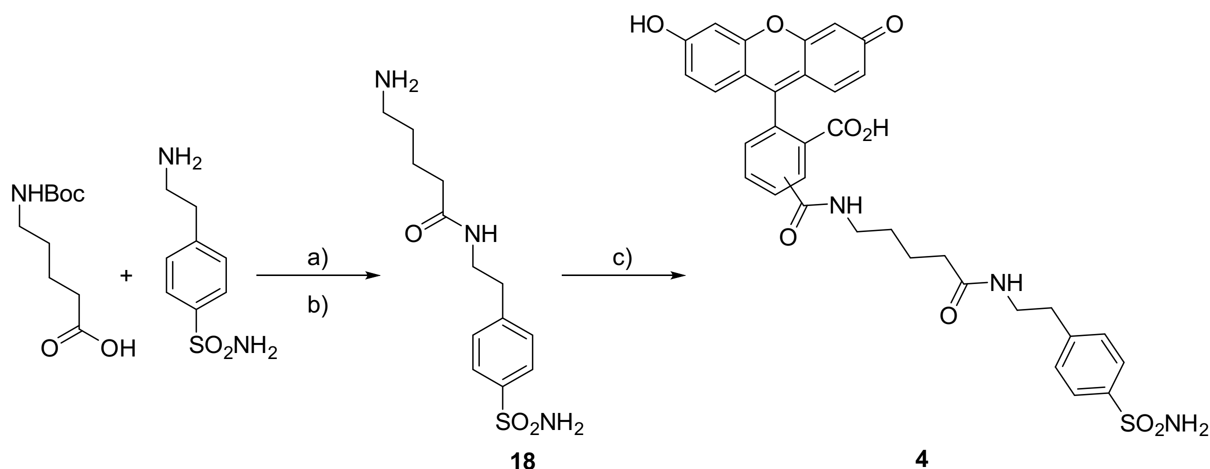
Scheme S5: a) DCC, HOBt, EDCI·HCl, DIPEA, DMF, 70 °C, 18 h, 83% b) $[IrCp^*Cl_2]_2$, EtOH, 80 °C, 2 h, 35%.

Compound 16 was synthesized according to a reported procedure.[6]

Ligand 17. 4-hydroxypicolinic acid (50 mg, 0.36 mmol), HOBt (100 mg, 0.74 mmol), EDCI·HCl (140 mg, 0.73 mmol) and DIPEA (125 µL, 0.76 mmol) were added to a solution of **16** (123 mg, 0.54 mmol) in DMF (3 mL). The solution was heated at 70 °C for 18 h. The solvents were removed *in vacuo*. Purification of the residue using silica gel column chromatography (CH_2Cl_2/MeOH 90/10) gave **17** (105 mg, 83%) as a white solid. R_f (CH_2Cl_2/MeOH 90/10): 0.10; ^{1}H NMR (500 MHz, DMSO-*d6*): 11.10 (bs, 1H), 10.43 (s, 1H), 8.93 (bs, 1H), 8.36 (bs, 1H), 7.78-7.73 (m, 4H), 7.44 (bs, 1H), 7.25 (s, 1H), 6.95 (bs, 1H), 4.14 (d, $^3J_{H-H}$ = 5.9 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-*d6*): 168.0, 165.2, 164.2, 151.3, 149.9, 141.7, 138.4, 126.8, 118.7, 113.9, 109.5, 43.0 ; MS (ESI, +ve): 351 (100, $[M+H]^+$); HRMS (ESI, +ve) calcd for $C_{14}H_{15}N_4O_5S$ ($[M+H]^+$): 351.0758, found: 351.0757.

Cofactor 10. The $[IrCp^*Cl_2]_2$ precursor (25 mg, 0.03 mmol) was added under N_2 to a solution of ligand **17** (20 mg, 0.06 mmol) in EtOH (5 mL). The solution was heated at 80 °C for 2 h. The solvents were removed *in vacuo*. The remaining solid was triturated in DCM three times. Purification of the residue using silica gel column chromatography (CH_2Cl_2/MeOH 97/3) gave cofactor **10** (15 mg, 35%) as a yellow solid. R_f (CH_2Cl_2/MeOH 90/10): 0.13; ^{1}H NMR (500 MHz, DMSO-*d6*): 11.94 (bs, 1H), 9.88 (s, 1H), 8.45 (d, $^3J_{H-H}$ = 6.3 Hz, 1H), 7.79 (d, $^3J_{H-H}$ = 8.7 Hz, 2H), 7.72 (d, $^3J_{H-H}$ = 8.7 Hz, 2H), 7.22 (s, 2H), 7.20 (d, $^3J_{H-H}$ = 2.8 Hz, 1H), 7.07 (dd, $^4J_{H-H}$ = 2.8 Hz, $^3J_{H-H}$

= 6.3 Hz 1H), 4.84 (d, $^2J_{H-H}$ = 16.6 Hz, 1H), 4.00 (d, $^2J_{H-H}$ = 16.6 Hz, 1H), 1.63 (s, 15 H); ^{13}C NMR (126 MHz, DMSO-d_6): 172.1, 170.3, 166.5, 154.1, 151.6, 141.7, 138.3, 126.6, 118.5, 115.7, 112.2, 94.5, 86.2, 54.7, 8.5; MS (ESI, +ve): 677 (100, [M-Cl]$^+$); HRMS (ESI, +ve) calcd for $C_{24}H_{28}IrN_4O_5S$ ([M-Cl]$^+$): 677.1403, found: 677.1401.



Scheme S6: a) HATU, *N*-Boc-pentanoic acid, Et_3N, DMF, rt, 18 h, b) TFA, DCM, rt, 1 h, 99% over 2 steps, c) HATU, Et_3N, DMF, rt, 2 h, 25%.

Compound 18. HATU (200 mg, 0.53 mmol), 4-(2-aminoethyl)benzenesulfonamide (108 mg, 0.49 mmol) and Et_3N (70 µL) were added to a solution of 5-[(*tert*-butoxycarbonyl)amino]pentanoic acid (100 mg, 0.49 mmol) in DMF (5 mL). The reaction was stirred at room temperature for 18 h. The solvents were removed under reduced pressure. The remaining solid was dissolved in a solvent mixture (DCM/MeOH 9/1, 10 ml) and Et_2O (40 ml) was added to precipitate a white solid. The solid was filtered. It was then suspended in DCM (5 mL), to which TFA (1mL) was added. After 1 h, the solvents were removed under reduced pressure to afford the desired compound **18** as a TFA salt (200 mg, 99%). ^{1}H NMR (500 MHz, MeOD): 7.83 (d, $^3J_{H-H}$ = 8.3 Hz, 2H), 7.40 (d, $^3J_{H-H}$ = 8.3 Hz, 2H), 3.47 (t, $^3J_{H-H}$ = 7.1 Hz, 2H), 2.89 (7, $^3J_{H-H}$ = 7.1 Hz, 4H), 2.20 (t, $^3J_{H-H}$ = 7.1 Hz, 2H), 1.64-1.52 (m, 4H); ^{13}C NMR (126 MHz, MeOD): 175.2, 145.3, 143.1, 130.5, 127.3, 41.2, 40.3, 36.3, 35.9, 28.0, 23.4; MS (ESI, +ve): 300 (100, [M+H]$^+$); HRMS (ESI, +ve) calcd for $C_{13}H_{22}IrN_3O_3S$ ([M+H]$^+$): 300.1376, found: 300.1376.

Fluorescent probe 4. HATU (50 mg, 0.13 mmol), **18** (50 mg, 0.12 mmol) and Et_3N (20 µL) were added to a solution of 5(6)-carboxyfluorescein (45 mg, 0.12 mmol) in DMF (5 mL). The reaction was stirred at room temperature for 2 h. The solvents were removed under reduced pressure. The residue was filtered using silica gel column chromatography (CH_2Cl_2/MeOH 90/10). Then a second purification using preparative thin layer chromatography (CH_2Cl_2/MeOH 85/15) was performed, followed by trituration in water. The resulting solid was dissolved in MeOH, dried over Na_2SO_4, and evaporated under reduced pressure to afford fluorescent probe **4** as an orange solid (20 mg, 25%). R_f (CH_2Cl_2/MeOH 90/10): 0.07; ^{1}H NMR (500 MHz, MeOD): 8.43 (s, 1H), 8.21-8.07 (m, 1H), 7.83-7.77 (m, 2H), 7.42-7.29 (m, 3H), 6.69 (s, 2H), 6.65-6.58 (m, 2H), 6.57-6.58 (m, 2H), 3.49-3.38 (m, 4H), 2.92-2.82 (m, 2H), 2.23-2.11 (m, 2H), 1.69-1.48 (m, 4H); MS (ESI, +ve): 658 (100, $[M+H]^+$); HRMS (ESI, +ve) calcd for $C_{34}H_{32}N_3O_9S$ ($[M+H]^+$): 658.1854, found: 658.1854.

e. Determination of the cofactor's 6-10 dissociation constant K_d.

$K_{d(DNSA)}$ was determined using the previously described procedure.[7] In a 96-well plate, CAII-DNSA stock solution (CAII: 0.1 µM, DNSA 10 µM, MOPS buffer pH 7.4, 10% v/v DMSO, 200 µL) were transferred into 17 wells. Aliquots (from 1 µL to 510 µL) of cofactor solutions **6**-**10** (10 µM) prepared with the stock solution of CAII-DNSA were added. The solutions were incubated for 18 h. An aliquot (150 µL) of each solution was transferred in a black flat-bottom 96-well microtiter plate (Nunclon) and mixed for 5 min at 25 °C in an Infinite M1000 Pro plate reader (Tecan). The fluorescence was determined with an excitation at 280 nm and emission at 470 nm. To determine the cofactor K_d, relative fluorescence r of each sample was determined using equation:

$$r = \frac{\mathrm{Fobs} - \mathrm{Fmin}}{\mathrm{Fmax} - \mathrm{Fmin}}$$

The relative fluorescence r was plotted against the relative cofactor concentration [cofactor]/[DNSA] and the K_d was obtained through fitting the following equation in the SIGMAPLOT program:

$$f = \frac{1}{(1 + \left(\frac{K_{d(DNSA)}}{[\mathrm{DNSA}]}\right) * \ 1 + \left(\frac{[\mathrm{cofactor}]}{K_d}\right)}$$

were $K_{d(DNSA)}$ and [DNSA] are the dissociation constant of DNSA for CAII and the DNSA concentration, respectively.

For cofactor **6**, no dissociation curve could be obtained from the data points suggesting that $K_{d(\mathbf{6})}$ is higher than $K_{d(DNSA)}$.

f. Transfer hydrogenation assays and ICP-MS sample preparation

Upon CAII expression (see above), cells were harvested into 96-well plates by transferring a solution of OD_{600} 3/mL cells into separate wells. The plates for the activity assays and ICP-MS analysis were handled in parallel. The activity assays were carried out in triplicate and ICP-MS samples were determined in triplicate. For one ICP-MS sample, 24 wells containing cells with OD_{600} 3/mL were pooled. The plates were centrifuged (3200 g, 4 ˚C, 8 min) and the supernatant was discarded. All cell pellets were resuspended in washing buffer (1 mL, 0.1 M MOPS, 154 mM NaCl, pH 7.4 supplemented with 0.8 mM [Cu(gly)$_2$]), centrifuged and the supernatant was discarded. The cell pellets were resuspended cofactor buffer (0.25 mL, 0.1 M MOPS, 154 mM NaCl, pH 7.4, 2 µM cofactor **5**-**10**) and incubated (1 h at 10 °C, shaking at 280 rpm). After a cofactor titration assay with cofactor **7,** 2 µM cofactor concentration was chosen (Figure S3). For cofactor concentrations higher than 2 µM, the background umbelliferone formation of the cells containing an empty vector increased significantly. The plates were centrifuged and the supernatant was discarded, followed by a second and third wash with washing buffer (1 mL, 0.1 M MOPS, 154 mM NaCl, pH 7.4).

For catalysis, the cells were resuspended in catalysis buffer (0.25 mL, 1 M sodium formate. 0.4 M MOPS, 154 mM NaCl and 0.5 mM substrate **1** pH 7.4). The plate was incubated shaking (16 h at 30 °C, 280 rpm). After completion of catalysis, the plate was centrifuged (3200 g, 4 ˚C, 8 min) and the supernatant (5 µL) was transferred to a black 96-well plate containing ddH_2O (245 µL) for fluorescence determination. The fluorescence was determined (323 nm excitation and 451 nm emission) using a microtiter plate reader and the conversion was determined using a calibration curve (Figure S2).

For inductively-coupled plasma mass-spectrometry (ICP-MS) analysis, 24 wells were pooled after the third washing step and resuspended in ddH_2O (250 µL). The ICP-MS analysis was performed by Solvias AG, Switzerland using an Agilent ICP-MS 7900#100 instrument.

g. Labelling of CAII expressing cells with fluorescent probe 4

Upon CAII expression (see above), the cell density was normalized to OD600 = 1. The normalized culture samples (0.2 mL) were transferred to reaction tubes (1.5 mL), spun down (14.000 g, 1 min) and the supernatant was discarded. The pellets were resuspended in washing buffer (0.9 mL, 0.1 M MOPS, 154 mM NaCl, pH 7.4 supplemented with [$Cu(gly)_2$] (0.8 mM), centrifuged (14.000 g, 30 s) and the supernatant was discarded. The pellets were resuspended in probe **4** solution (0.1 mL, 10 µM probe 4, 0.1 M MOPS, 154 mM NaCl, pH 7.4) and incubated (30 min, RT, at 500 rpm). After centrifugation (14.000 g, 30 s) the pellets were resuspended in washing buffer (0.9 mL, 0.1 M MOPS, 154 mM NaCl, pH 7.4), centrifuged (14.000 g, 30 s) and the supernatant was discarded. The pellets were resuspended for analysis in washing buffer (10 µL or 5 mL, 0.1 M MOPS, 154 mM NaCl, pH 7.4 for microscopy or flow cytometry analysis, respectively).

h. Labelling of CAII expressing cells with anti-CAII antibodies

Upon CAII expression (see above), the cell density was normalized to OD_{600} = 1. The normalized cultures (0.2 mL) were transferred to a reaction tubes (1.5 mL), spun-down (14.000 g, 1 min) and the supernatant was discarded. The pellets were resuspended in ice-cold TBSF (0.9 mL, 25 mM Tris, 154 mM NaCl, 0.25 % (w/v) bovine serum albumin (BSA), pH 7.5) centrifuged (14.000 g, 30 s) and the supernatant was discarded. The pellets were resuspended in primary rabbit anti-CAII antibody solution (0.2 mL, 1:100 dilution primary antibody [ab191343, abcam, Cambridge, USA] in TBSF) and incubated (30 min at RT, 500 rpm). After centrifugation (14.000 g, 30 s), the pellets were resuspended in ice-cold TBSF (0.9 mL) centrifuged (14.000 g, 30 s) and the supernatant was discarded. The pellets were resuspended in buffer (0.2 mL) containing fluorescent (Alexa Fluor® 488) secondary goat anti-rabbit antibody (1:250 dilution secondary antibody [ab150077, abcam, Cambridge, USA] in TBSF) and incubated on ice (20 min). After centrifugation (14.000 g, 30 s) the pellets were washed with ice-cold TBSF (0.9 mL). The pellets were resuspended in TBSF (10 µL or 5 mL for microscopy or flow cytometry analysis, respectively).

i. Microscopy analysis of probe 4 and antibody-labelled *E. coli* cells

To visualize the stained cells confocal laser scanning microscopy (Zeiss LSM 880) was performed using an Argon (λ = 488 nm, 2 % laser intensity) laser and an oil immersion objective (Pln-Apochromat 63×/1.4 Oil). The pinhole diameter (51.5 µm), main beam splitter (488) and a bandpass filter (489 – 601 nm) were used throughout all experiments. All images were recorded with a fixed detector gain (900 for fluorescence and 320 for brightfield). The same brightness and contrast were applied to all images using ImageJ.

j. Flow-cytometry analysis of probe 4 and antibody-labelled *E. coli* cells

The probe **4** and antibody-labelled cells were analyzed by flow cytometry using an Attune NxT cell analyzer equipped with a blue laser (488 nm) and a filter (530/30 nm). The samples were measured (flow rate of 12.5 µL/min) and histograms are displayed in (Figure 2c, d) and comprise data of 200,000 ungated events.

k. Crystal structure solution and analysis of CAII soaked with cofactor 7-10

CAII crystallization, crystal stabilization and cofactor soaking. CAII was expressed and purified as described previously.[4, 7-9] Lyophilized wild-type CAII was dissolved in ultrapure water (18.2 MΩ·cm, MilliQ, Millipore Corporation, Burlington, USA). For sitting drop vapor diffusion, CAII (4.5 µL 26 mg/mL) was mixed with precipitation buffer (0.5 µL, 2.6 M $(NH_4)_2SO_4$, 50 mM Tris-H_2SO_4, pH 7.9). The drop was equilibrated against a reservoir of precipitation buffer (100 µL at 20 °C). Crystal plates of apo-CAII crystals (just containing a zinc in the active site) grew within five days. To stabilize the crystals for soaking, a glutaraldehyde crosslinking was performed.[7] In a 24-well sitting drop crystallization plate with a central column precipitation buffer were pipetted into the reservoir and the sitting-drop depression (500 µL and 5 µL). The crystals were transferred into the sitting-drop depression and the well was sealed with a cover slide having a hanging drop of 25 % glutaraldehyde solution (5 µL) and equilibrated (20 °C, 5 h). For soaking, glutaraldehyde-stabilized crystals were individually transferred into sitting-drop depressions containing precipitation buffer (2.375 µL) and cofactor solution (0.125 µL, 10 mM cofactor **7**-**10** in DMSO). After soaking (2.5 h, 20 °C) the crystals were flash-frozen in liquid nitrogen.

Diffraction data collection and processing. Protein-crystal diffraction data were collected (100 K) at the Swiss Light Source beam line PSI at a wavelength of 1.0 Å. Crystal indexing, integration and scaling[10, 11] were carried out with the program XDS[12] and Dials[13] in Xia2[10, 14] using the graphical interface CCP4i2[15] of the CCP4 suite[16] (see Table S6 for processing statistics).

Structure solution and refinement. The structure was solved by molecular replacement using the program PHASER MR[17] and the wild type CAII structure PDB: 3ZP9, devoid of the Ir-cofactor and water molecules. For structure refinement, REFMAC[18, 19] of the CCP4 Suite was used. For structure modelling, water picking and electron-density visualization the software COOT[20] was used. Figures were prepared with PyMOL (the PyMOL Molecular Graphics System, Version 1.821, Schrödinger, LLC).

A single molecule CAII was obtained per asymmetric unit. Amino acid residues 1-3 and 260 at the beginning and the end of the protein are not resolved in the electron density, presumably due to disorder.

Structure refinement of iridium cofactor 7-10. Residual electron density in the Fo-Fc map was observed in the active site of CAII. Anomalous dispersion density was observed in the CAII vestibule for the Zn-atom coordinated by His94, His96 and His114 and further up in the binding pocket. Modelling of cofactor **7**-**10** into the electron density projected the iridium in the position of the anomalous density peak. Although cofactor **7** and **8** have the same linker and only differ by the alcohol group on the picolinamide *para* to the nitrogen of cofactor **7**, they adopt a slightly different position in the binding pocket of CAII (see Figure S6). Compared to cofactor **7,** cofactor **8** is shifted 2 Å closer to the α-helix of loop 4,5. To accommodate this, F130 moves slightly out of the CAII pocket to allow π–stacking with cofactor **8**. Furthermore, E69 of CAII bound to the cofactor **7** moves 1.9 Å closer to the cofactor. We hypothesize that this may contribute to stabilize the bound cofactor **7**.

2. Supporting Tables

Table S1: Plasmids used in this study.

Name	Description	Source/Reference
Empty Vector	pET-30b(+), pBR322 ori, Kan^R, P_{T7} promotor	Merck Millipore, Darmstadt Germany
$CAII^{cytoplasm}$	F1 ori, Amp^R, P_{T7} promotor. Source of CAII gene	Carol Fierke, Michigan University[21]
$CAII^{periplasm}$	pET-30b(+), pBR322 ori, Kan^R, P_{T7} promotor. With gene of OmpA-CAII fusion for periplasmic expression of CAII	This study
$CAII^{surface\ display}$	pET-30b(+), pBR322 ori, Kan^R, P_{T7} promotor. With gene of Lpp-OmpA-CAII fusion for surface display of CAII	This study
pET-30b_Sav^{peri}	pET-30b(+) with gene for an OmpA-SAV fusion protein for periplasmic expression of Sav. Sav was replaced with CAII to create the plasmid "Periplasmic CAII"	Jeschek *et al.,* 2016[1]
pET-30b_Lpp-OmpA-Sav_{WT}	pET-30b(+) with gene for a Lpp-OmpA-SAV fusion protein for surface display of Sav. Sav was replaced with CAII to create the plasmid "Surface CAII"	Heinisch *et al.*, 2018[2]

Table S2: Primers used for cloning.

Primer	Sequence (5'-3')
NheI-CAII_fwd	GACTAGCTAGCATGGCCCATCACTGGGGGTAC
SacI-CAII_rev	GCACGAGCTCGGGACCATCTTATTTGAAGGAAGC

Table S3: DNA sequence of the plasmid "periplasmic CAII"

tggcgaatgggacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgc

cctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaaatcgggggctccctttagggttc

cgatttagtgctttacggcacctcgaccccaaaaaacttgattagggtgatggttcacgtagtgggccatcgccctgatagacggtttttcgc

cctttgacgttggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcggtctattcttttgatttataag

ggattttgccgatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaacgcgaattttaacaaaatattaacgtttacaatttca

ggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgaattaattcttagaa

aaactcatcgagcatcaaatgaaactgcaatttattcatatcaggattatcaataccatatttttgaaaaagccgtttctgtaatgaaggaga

aaactcaccgaggcagttccataggatggcaagatcctggtatcggtctgcgattccgactcgtccaacatcaatacaacctattaatttc

ccctcgtcaaaaataaggttatcaagtgagaaatcaccatgagtgacgactgaatccggtgagaatggcaaaagtttatgcatttctttcc

agacttgttcaacaggccagccattacgctcgtcatcaaaatcactcgcatcaaccaaaccgttattcattcgtgattgcgcctgagcgag

acgaaatacgcgatcgctgttaaaaggacaattacaaacaggaatcgaatgcaaccggcgcaggaacactgccagcgcatcaaca

atattttcacctgaatcaggatattcttctaatacctggaatgctgttttcccggggatcgcagtggtgagtaaccatgcatcatcaggagtac

ggataaaatgcttgatggtcggaagaggcataaattccgtcagccagtttagtctgaccatctcatctgtaacatcattggcaacgctacct

ttgccatgtttcagaaacaactctggcgcatcgggcttcccatacaatcgatagattgtcgcacctgattgcccgacattatcgcgagccca

tttatacccatataaatcagcatccatgttggaatttaatcgcggcctagagcaagacgtttcccgttgaatatggctcataacaccccttgta

ttactgtttatgtaagcagacagttttattgttcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaag

atcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccgg

atcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggc

caccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtctt

accgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacggggggttcgtgcacacagcccagcttgga

gcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacag

gtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgg

gtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggcctttttac

ggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgata

ccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcctgatgcggtattttctccttacg

catctgtgcggtatttcacaccgcatatatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatacactccgctatc

gctacgtgactgggtcatggctgcgccccgacacccgccaacacccgctgacgcgccctgacgggcttgtctgctcccggcatccgctt

acagacaagctgtgaccgtctccgggagctgcatgtgtcagaggttttcaccgtcatcaccgaaacgcgcgaggcagctgcggtaaag

ctcatcagcgtggtcgtgaagcgattcacagatgtctgcctgttcatccgcgtccagctcgttgagtttctccagaagcgttaatgtctggctt

ctgataaagcgggccatgttaagggcggttttttcctgtttggtcactgatgcctccgtgtaagggggatttctgttcatgggggtaatgatac

cgatgaaacgagagaggatgctcacgatacgggttactgatgatgaacatgcccggttactggaacgttgtgagggtaaacaactggc

ggtatggatgcggcgggaccagagaaaaatcactcagggtcaatgccagcgcttcgttaatacagatgtaggtgttccacagggtagc

cagcagcatcctgcgatgcagatccggaacataatggtgcagggcgctgacttccgcgtttccagactttacgaaacacggaaaccga

agaccattcatgttgttgctcaggtcgcagacgttttgcagcagcagtcgcttcacgttcgctcgcgtatcggtgattcattctgctaaccagt

aaggcaaccccgccagcctagccgggtcctcaacgacaggagcacgatcatgcgcacccgtggggccgccatgccggcgataatg

gcctgcttctcgccgaaacgtttggtggcgggaccagtgacgaaggcttgagcgagggcgtgcaagattccgaataccgcaagcgac

aggccgatcatcgtcgcgctccagcgaaagcggtcctcgccgaaaatgacccagagcgctgccggcacctgtcctacgagttgcatg

ataaagaagacagtcataagtgcggcgacgatagtcatgccccgcgcccaccggaaggagctgactgggttgaaggctctcaaggg
catcggtcgagatcccggtgcctaatgagtgagctaacttacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgt
gccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgccagggtggtttttcttttcaccagtgagacg
ggcaacagctgattgcccttcaccgcctggccctgagagagttgcagcaagcggtccacgctggtttgccccagcaggcgaaaatcct
gtttgatggtggttaacggcgggatataacatgagctgtcttcggtatcgtcgtatcccactaccgagatgtccgcaccaacgcgcagccc
ggactcggtaatggcgcgcattgcgcccagcgccatctgatcgttggcaaccagcatcgcagtgggaacgatgccctcattcagcattt
gcatggtttgttgaaaaccggacatggcactccagtcgccttcccgttccgctatcggctgaatttgattgcgagtgagatatttatgccagc
cagccagacgcagacgcgccgagacagaacttaatgggcccgctaacagcgcgatttgctggtgacccaatgcgaccagatgctcc
acgcccagtcgcgtaccgtcttcatgggagaaaataatactgttgatgggtgtctggtcagagacatcaagaaataacgccggaacatt
agtgcaggcagcttccacagcaatggcatcctggtcatccagcggatagttaatgatcagcccactgacgcgttgcgcgagaagattgt
gcaccgccgctttacaggcttcgacgccgcttcgttctaccatcgacaccaccacgctggcacccagttgatcggcgcgagatttaatcg
ccgcgacaatttgcgacggcgcgtgcagggccagactggaggtggcaacgccaatcagcaacgactgtttgcccgccagttgttgtgc
cacgcggttgggaatgtaattcagctccgccatcgccgcttccactttttcccgcgttttcgcagaaacgtggctggcctggttcaccacgc
gggaaacggtctgataagagacaccggcatactctgcgacatcgtataacgttactggtttcacattcaccaccctgaattgactctcttcc
gggcgctatcatgccataccgcgaaaggttttgcgccattcgatggtgtccgggatctcgacgctctcccttatgcgactcctgcattagga
agcagcccagtagtaggttgaggccgttgagcaccgccgccgcaaggaatggtgcatgcaaggagatggcgcccaacagtccccc
ggccacggggcctgccaccatacccacgccgaaacaagcgctcatgagcccgaagtggcgagcccgatcttccccatcggtgatgtc
ggcgatataggcgccagcaaccgcacctgtggcgccggtgatgccggccacgatgcgtccggcgtagaggatcgagatcgatctcg
atcccgcgaaattaatacgactcactataggggaattgtgagcggataacaattcccctctagaaataattttgtttaactttaagaaggag
atataCATATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAG
CGCAGGCCGCTAGCATGGCCCATCACTGGGGGTACGGCAAACACAACGGACCTGAGCACT
GGCATAAGGACTTCCCCATTGCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCA
TACAGCCAAGTATGACCCTTCCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCC
TGAGGATCCTCAACAATGGTCATGCTTTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCA
GTGCTCAAGGGAGGACCCCTGGATGGTACCTACAGATTGATTCAGTTTCACTTTCACTGGGG
TTCACTTGATGGCCAAGGTTCAGAGCATACTGTGGATAAAAAGAAATATGCTGCAGAACTTC
ACTTGGTTCACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCAGCAACCTGATGGA
CTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCTAAACCGGGCCTTCAGAAAGTTGT
TGATGTGCTGGATTCCATTAAAACAAAGGGCAAGAGTGCTGACTTCACTAACTTCGATCCTC

GTGGCCTCCTTCCTGAATCCCTGGATTACTGGACCTACCCAGGCTCACTGACCACCCCTCCT
CTTCTGGAATGTGTGACCTGGATTGTGCTCAAGGAACCCATCAGCGTCAGCAGCGAGCAGG
TGTTGAAATTCCGTAAACTTAACTTCAATGGGGAGGGTGAACCCGAAGAACTGATGGTGGAC
AACTGGCGCCCAGCTCAGCCACTGAAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGG
TCCCGAGCTccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccggctgctaacaaagcccg
aaaggaagctgagttggctgctgccaccgctgagcaataactagcataaccccttggggcctctaaacgggtcttgaggggttttttgctg
aaaggaggaactatatccggat

grey: OmpA signal peptide, blue: CAII, red: restriction site NheI, violet: restriction site SacI

Table S4: DNA sequence of the plasmid "surface CAII"

tggcgaatgggacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgc
cctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaaatcgggggctccctttagggttc
cgatttagtgctttacggcacctcgaccccaaaaaacttgattagggtgatggttcacgtagtgggccatcgccctgatagacggtttttcgc
cctttgacgttggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcggtctattcttttgatttataag
ggattttgccgatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaacgcgaattttaacaaaatattaacgtttacaatttca
ggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgaattaattcttagaa
aaactcatcgagcatcaaatgaaactgcaatttattcatatcaggattatcaataccatatttttgaaaaagccgtttctgtaatgaaggaga
aaactcaccgaggcagttccataggatggcaagatcctggtatcggtctgcgattccgactcgtccaacatcaatacaacctattaatttc
ccctcgtcaaaaataaggttatcaagtgagaaatcaccatgagtgacgactgaatccggtgagaatggcaaaagtttatgcatttctttcc
agacttgttcaacaggccagccattacgctcgtcatcaaaatcactcgcatcaaccaaaccgttattcattcgtgattgcgcctgagcgag
acgaaatacgcgatcgctgttaaaaggacaattacaaacaggaatcgaatgcaaccggcgcaggaacactgccagcgcatcaaca
atattttcacctgaatcaggatattcttctaatacctggaatgctgttttcccggggatcgcagtggtgagtaaccatgcatcatcaggagtac
ggataaaatgcttgatggtcggaagaggcataaattccgtcagccagtttagtctgaccatctcatctgtaacatcattggcaacgctacct
ttgccatgtttcagaaacaactctggcgcatcgggcttcccatacaatcgatagattgtcgcacctgattgcccgacattatcgcgagccca
tttatacccatataaatcagcatccatgttggaatttaatcgcggcctagagcaagacgtttcccgttgaatatggctcataacaccccttgta
ttactgtttatgtaagcagacagttttattgttcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaag
atcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccgg
atcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggc
caccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtctt

accgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacggggggttcgtgcacacagcccagcttgga
gcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacag
gtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgg
gtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggcctttttac
ggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgata
ccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcctgatgcggtattttctccttacg
catctgtgcggtatttcacaccgcatatatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatacactccgctatc
gctacgtgactgggtcatggctgcgccccgacacccgccaacacccgctgacgcgccctgacgggcttgtctgctcccggcatccgctt
acagacaagctgtgaccgtctccgggagctgcatgtgtcagaggttttcaccgtcatcaccgaaacgcgcgaggcagctgcggtaaag
ctcatcagcgtggtcgtgaagcgattcacagatgtctgcctgttcatccgcgtccagctcgttgagtttctccagaagcgttaatgtctggctt
ctgataaagcgggccatgttaagggcggttttttcctgtttggtcactgatgcctccgtgtaagggggatttctgttcatgggggtaatgatac
cgatgaaacgagagaggatgctcacgatacgggttactgatgatgaacatgcccggttactggaacgttgtgagggtaaacaactggc
ggtatggatgcggcgggaccagagaaaaatcactcagggtcaatgccagcgcttcgttaatacagatgtaggtgttccacagggtagc
cagcagcatcctgcgatgcagatccggaacataatggtgcagggcgctgacttccgcgtttccagactttacgaaacacggaaaccga
agaccattcatgttgttgctcaggtcgcagacgttttgcagcagcagtcgcttcacgttcgctcgcgtatcggtgattcattctgctaaccagt
aaggcaaccccgccagcctagccgggtcctcaacgacaggagcacgatcatgcgcacccgtggggccgccatgccggcgataatg
gcctgcttctcgccgaaacgtttggtggcgggaccagtgacgaaggcttgagcgagggcgtgcaagattccgaataccgcaagcgac
aggccgatcatcgtcgcgctccagcgaaagcggtcctcgccgaaaatgacccagagcgctgccggcacctgtcctacgagttgcatg
ataaagaagacagtcataagtgcggcgacgatagtcatgccccgcgcccaccggaaggagctgactgggttgaaggctctcaaggg
catcggtcgagatcccggtgcctaatgagtgagctaacttacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgt
gccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgccagggtggtttttcttttcaccagtgagacg
ggcaacagctgattgcccttcaccgcctggccctgagagagttgcagcaagcggtccacgctggtttgccccagcaggcgaaaatcct
gtttgatggtggttaacggcgggatataacatgagctgtcttcggtatcgtcgtatcccactaccgagatgtccgcaccaacgcgcagccc
ggactcggtaatggcgcgcattgcgcccagcgccatctgatcgttggcaaccagcatcgcagtgggaacgatgccctcattcagcattt
gcatggtttgttgaaaaccggacatggcactccagtcgccttcccgttccgctatcggctgaatttgattgcgagtgagatatttatgccagc
cagccagacgcagacgcgccgagacagaacttaatgggcccgctaacagcgcgatttgctggtgacccaatgcgaccagatgctcc
acgcccagtcgcgtaccgtcttcatgggagaaaataatactgttgatgggtgtctggtcagagacatcaagaaataacgccggaacatt
agtgcaggcagcttccacagcaatggcatcctggtcatccagcggatagttaatgatcagcccactgacgcgttgcgcgagaagattgt
gcaccgccgctttacaggcttcgacgccgcttcgttctaccatcgacaccaccacgctggcacccagttgatcggcgcgagatttaatcg

ccgcgacaatttgcgacggcgcgtgcagggccagactggaggtggcaacgccaatcagcaacgactgtttgcccgccagttgttgtgc
cacgcggttgggaatgtaattcagctccgccatcgccgcttccactttttcccgcgttttcgcagaaacgtggctggcctggttcaccacgc
gggaaacggtctgataagagacaccggcatactctgcgacatcgtataacgttactggtttcacattcaccaccctgaattgactctcttcc
gggcgctatcatgccataccgcgaaaggttttgcgccattcgatggtgtccgggatctcgacgctctcccttatgcgactcctgcattagga
agcagcccagtagtaggttgaggccgttgagcaccgccgccgcaaggaatggtgcatgcaaggagatggcgcccaacagtccccc
ggccacggggcctgccaccatacccacgccgaaacaagcgctcatgagcccgaagtggcgagcccgatcttccccatcggtgatgtc
ggcgatataggcgccagcaaccgcacctgtggcgccggtgatgccggccacgatgcgtccggcgtagaggatcgagatcgatctcg
atcccgcgaaattaatacgactcactataggggaattgtgagcggataacaattcccctctagaaggagatatacacttcatgaaagc
gaccaaactggtgctgggcgcggtgattctgggcagcaccctgctggcgggctgcagcagcaacgcgaaaattgatcagaacaaca
acggcccgacccatgaaaaccagctgggcgcgggcgcgtttggcggctatcaggtgaacccgtatgtgggctttgaaatgggctatga
ttggctgggccgcatgccgtataaaggcagcgtggaaaacggcgcgtataaagcgcagggcgtgcagctgaccgcgaaactgggct
atccgattaccgatgatctggatatttatacccgcctgggcggcatggtgtggcgcgcggataccaaaagcaacgtgtatggcaaaaac
catgataccggcgtgagcccggtgtttgcgggcggcgtggaatatgcgattaccccggaaattgcgaccggagCTAGCATGGC
CCATCACTGGGGGTACGGCAAACACAACGGACCTGAGCACTGGCATAAGGACTTCCCCATT
GCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCATACAGCCAAGTATGACCCTT
CCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGATCCTCAACAATGGT
CATGCTTTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCAGTGCTCAAGGGAGGACCCC
TGGATGGTACCTACAGATTGATTCAGTTTCACTTTCACTGGGGTTCACTTGATGGCCAAGGT
TCAGAGCATACTGTGGATAAAAAGAAATATGCTGCAGAACTTCACTTGGTTCACTGGAACAC
CAAATATGGGGATTTTGGGAAAGCTGTGCAGCAACCTGATGGACTGGCCGTTCTAGGTATTT
TTTTGAAGGTTGGCAGCGCTAAACCGGGCCTTCAGAAAGTTGTTGATGTGCTGGATTCCATT
AAAACAAAGGGCAAGAGTGCTGACTTCACTAACTTCGATCCTCGTGGCCTCCTTCCTGAATC
CCTGGATTACTGGACCTACCCAGGCTCACTGACCACCCCTCCTCTTCTGGAATGTGTGACCT
GGATTGTGCTCAAGGAACCCATCAGCGTCAGCAGCGAGCAGGTGTTGAAATTCCGTAAACT
TAACTTCAATGGGGAGGGTGAACCCGAAGAACTGATGGTGGACAACTGGCGCCCAGCTCAG
CCACTGAAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGGTCCCGAGCTccgtcgacaagct
tgcggccgcactcgagcaccaccaccaccaccactgagatccggctgctaacaaagcccgaaaggaagctgagttggctgctgcca
ccgctgagcaataactagcataaccccttggggcctctaaacgggtcttgaggggttttttgctgaaaggaggaactatatccggat

green: LPP, yellow: OmpA, blue: CAII, red: restriction site NheI, violet: restriction site SacI

Table S5: Modified ZYM-5052 rich medium

Component	Volume (mL)
Yeast-tryptone stock[a]	88.8
20x M-stock[b]	5
0.2 M $MgSO_4$	1
20 % maltose/10 % glucose + 10 % glycerol[c]	5
0.5 M $ZnSO_4$	0.1
100x antibiotic stock[d]	0.1
Total amount	100

[a] yeast-tryptone stock: 5 g yeast extract and 10 g tryptone in 1 L H_2O.

[b] 20x M-stock: 1 M Na_2HPO_4, 1 M KH_2PO_4, 0.5 M $(NH_4)_2SO_4$.

[c] 20 % maltose was used for the periplasmic expression and replaced by 10 % glucose for the other strains.

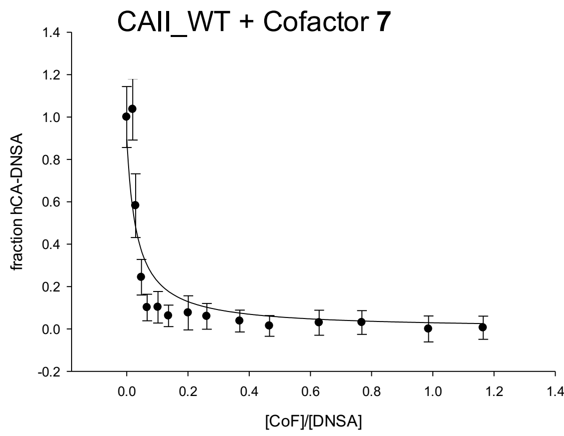
[d] 1000x antibiotic stock: 50 mg/mL kanamycin was used for the empty vector, periplasmic CAII and surface CAII and replaced by 100 mg/mL ampicillin for cytoplasmic CAII.

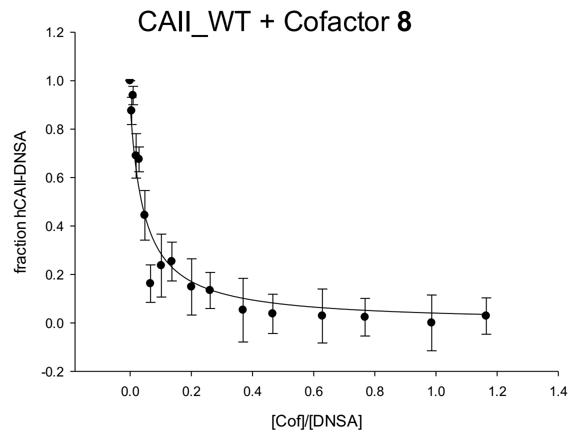
Table S6. Reflection data processing and crystal structure refinement statistics.

CAII	wild type	wild type	wild type	wild type
Ir-cofactor	Cofactor **7**	Cofactor **8**	Cofactor **9**	Cofactor **10**
PDB Code	6QFU	6QFV	6QFW	6QFX
Data Collection Statistics				
Wavelength (Å)	1.0000	1.0000	1.0000	1.0000
Resolution range (Å)	40.77 – 1.80 (1.85 – 1.80)[a]	41.68 – 1.45 (1.47 - 1.45)[a]	40.95 - 1.20 (1.23 - 1.20)[a]	41.02 - 1.32 (1.34 – 1.32)[a]
Cell Parameter - *a, b, c* (Å) - α, β, γ (°)	 42.0, 41.3, 71.9 90.0, 104.3, 90.0	 42.4, 41.7, 72.4 90.0, 104.4, 90.0	 42.2, 41.4, 72.4 90.0, 104.4, 90.0	 42.3, 41.6, 72.5 90.0, 104.4, 90.0
Space group	P 1 21 1	P 1 21 1	P 1 21 1	P 1 21 1
Unique reflections	21112 (1553)	43249 (2208)	70789 (5020)	55708 (3701)
Completeness (%)	99.3 (99.9)	96.7 (71.1)	98.0 (94.4)	95.9 (90.8)
Multiplicity	8.0 (8.5)	6.0 (3.1)	9.3 (6.4)	4.5 (4.5)
R_{merge} (%)	19.8 (102.6)	6.3 (47.3)	13.4 (20.0)	7.4 (106.9)
Mean I/Sig(I)	10.3 (7.0)	14.8 (2.8)	13.5 (7.0)	8.2 (1.2)
CC (1/2)	0.958 (0.386)	0.998 (0.488)	0.995 (0.975)	0.996 (0.350)
Structure Refinement Statistics				
R_{work}/R_{free}	0.165/0.187	0.156/0.168	0.162/0.178	0.172/0.197
RMS deviation - Bond length (Å) - Bond angles (°) - Ramachandran favored (%)	 0.0130 2.67 96.46	 0.0140 2.36 95.65	 0.0167 1.92 96.46	 0.0129 2.36 95.67
Average B-factors ($Å^2$) - Protein - Ligands - Water	 18.4 26.8 26.7	 19.8 21.7 30.1	 14.4 75.1 26.3	 23.1 38.2 34.1
Number ligand molecules - Zn - Ir-cofactor - Ir - sulfate - water	 1 1 0 1 157	 1 1 0 2 197	 1 1 1 1 268	 1 1 0 2 212
Anomalous dispersion density peaks (σ) - Zn - Ir-cofactor - Ir	 24.5 32.6 	 27.7 64.1 	 120 10.3 19.1	 53.3 26.9

[a] Values in parentheses represent for the highest resolution she

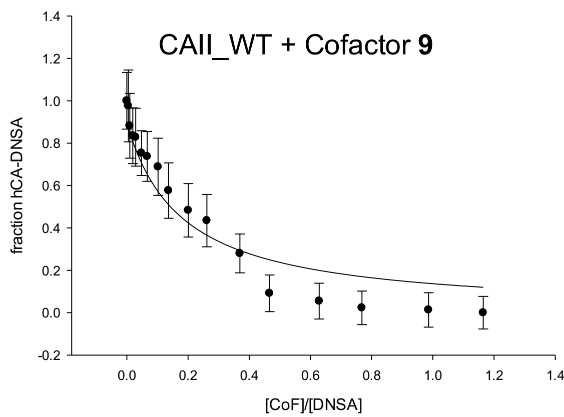
3. Supporting Figures

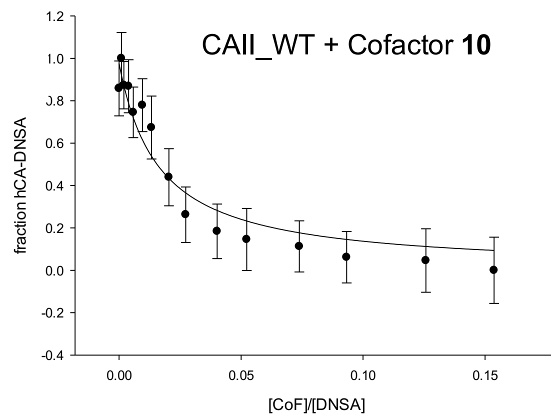




$R^2 = 0.81$; K_d = 35 nM ± 11

$R^2 = 0.94$; K_d = 49 nM ± 8





$R^2 = 0.92$; K_d = 149 nM ± 30

$R^2 = 0.93$; K_d = 20 nM ± 5

Figure S1. Competition assay of cofactor binding to CAII WT. Data displayed are the means, error bars are standard deviations of nine measurements.

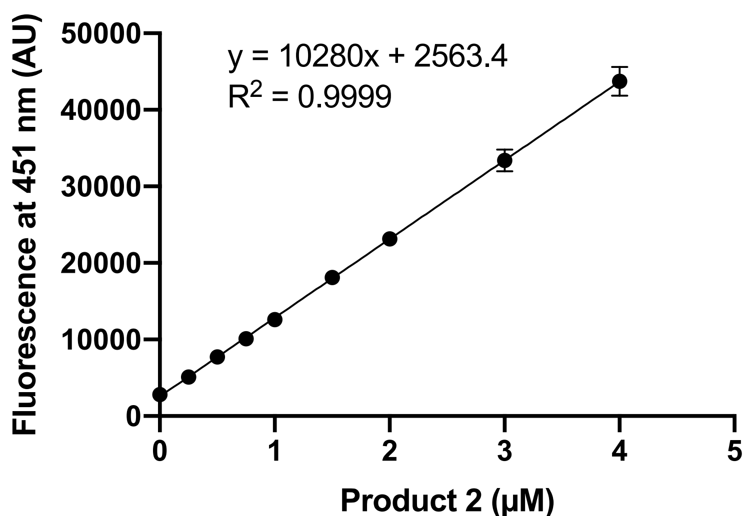


Figure S2. Calibration curve for fluorescence quantification of product **2**. Data displayed is the mean and the standard deviation of nine measurements and the resulting linear regression.

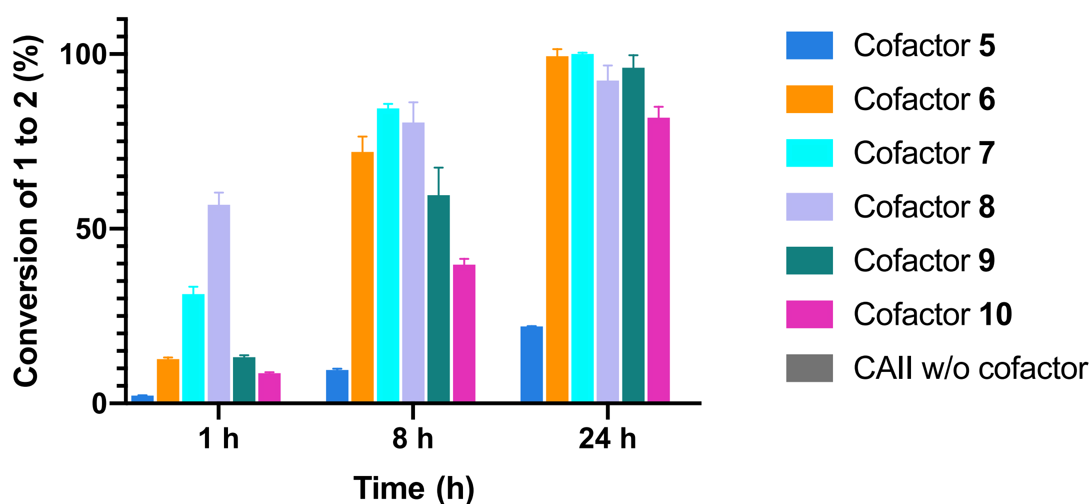


Figure S3. *In vitro* formation of umbelliferone **2** by cofactors **5** - **10** bound to CAII. The conversion of **1** to **2** was determined by fluorescence after 1, 8 and 24 h.

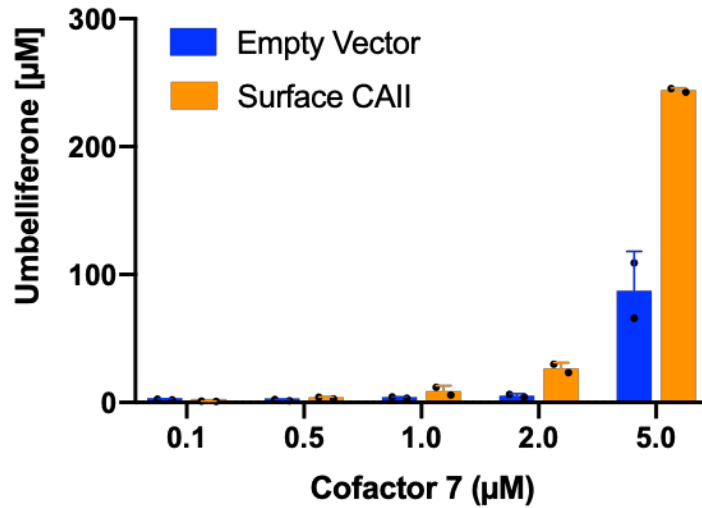


Figure S4. Testing the effect of the cofactor **7** concentration on the formation of umbelliferone. Assays with cells containing an empty vector or CAII$^{\text{surface display}}$ were incubated with different cofactor concentrations (0.1 – 5 µM). Data displayed are the means, error bars are standard deviations of experiments performed in duplicate, black dots are the individual data points.

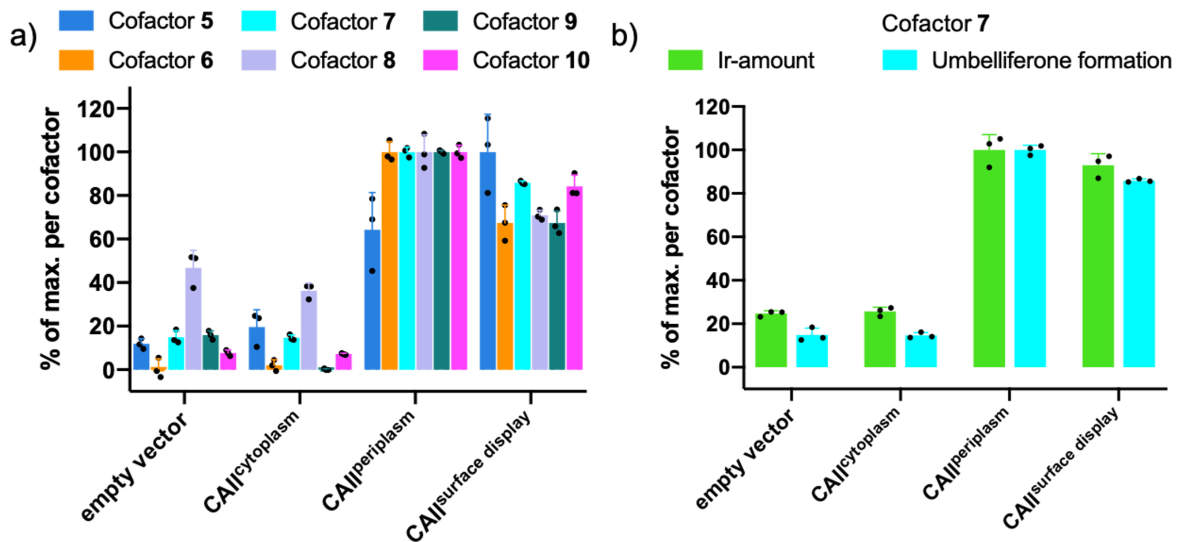


Figure S5. Cellular umbelliferone **2** formation and iridium enrichment. (a) The umbelliferone formation was normalized to the maximum activity for each cofactor. (b) The umbelliferone formation by cofactor **7** is compared to the Ir-amount measured after incubation with cofactor **7** for the different compartmentalization strategies of CAII: empty vector, CAII$^{\text{cytoplasm}}$, CAII$^{\text{periplasm}}$, CAII$^{\text{surface display}}$. Data displayed are the means, error bars are standard deviations of experiments performed in triplicate, black dots are the individual data points. There is no background subtracted.

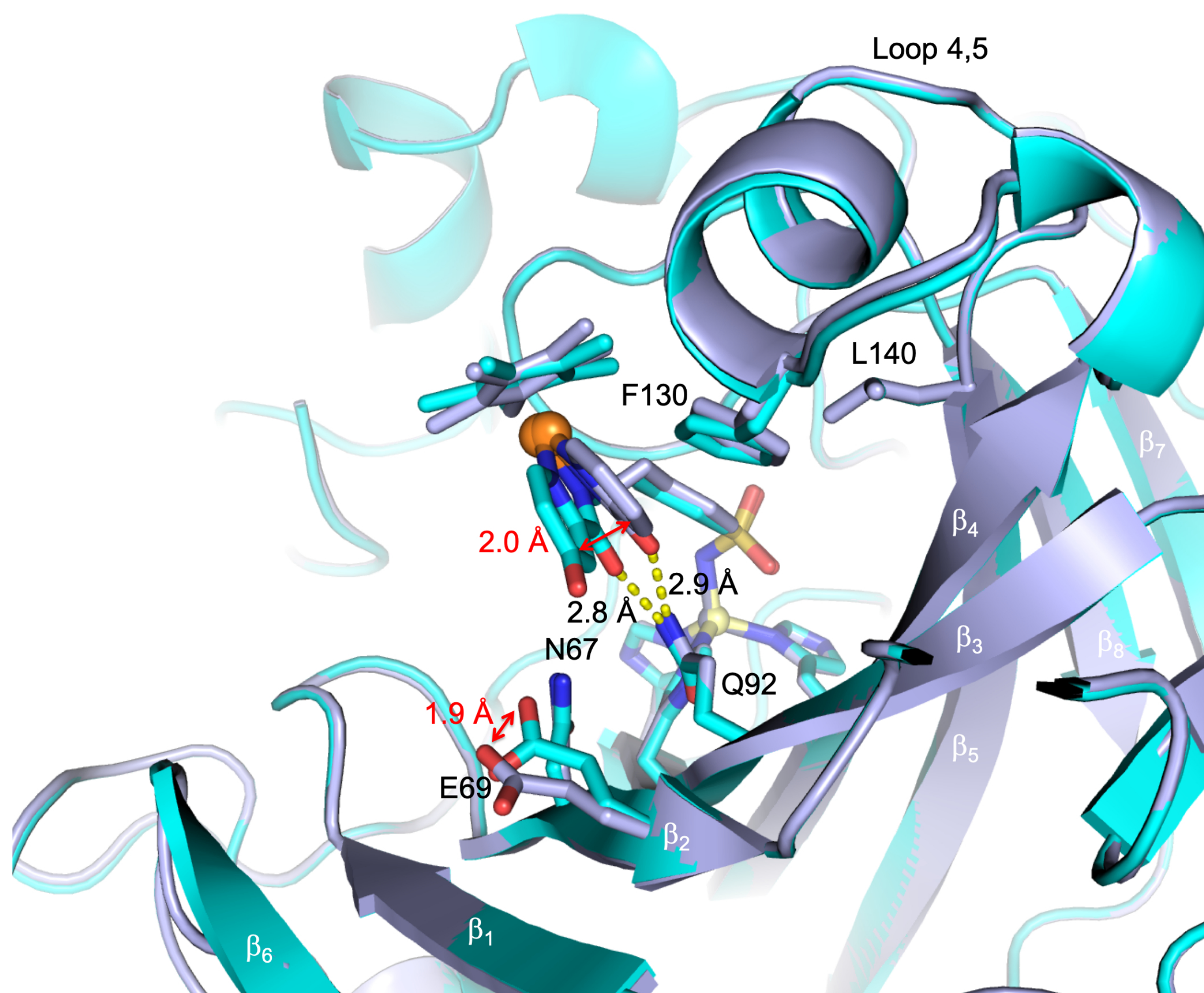


Figure S6. Comparison of the cofactor **7** and **8** binding to CAII. CAII is displayed as cartoon representation, CAII with bound cofactor **7** (in cyan) and with bound cofactor **8** (in lilac). Amino acids in the proximity of the cofactors are displayed as stick and labelled; nitrogen, blue; oxygen, red; sulfur, yellow. The iridium and zinc ions are shown as spheres – orange and pale yellow, respectively.

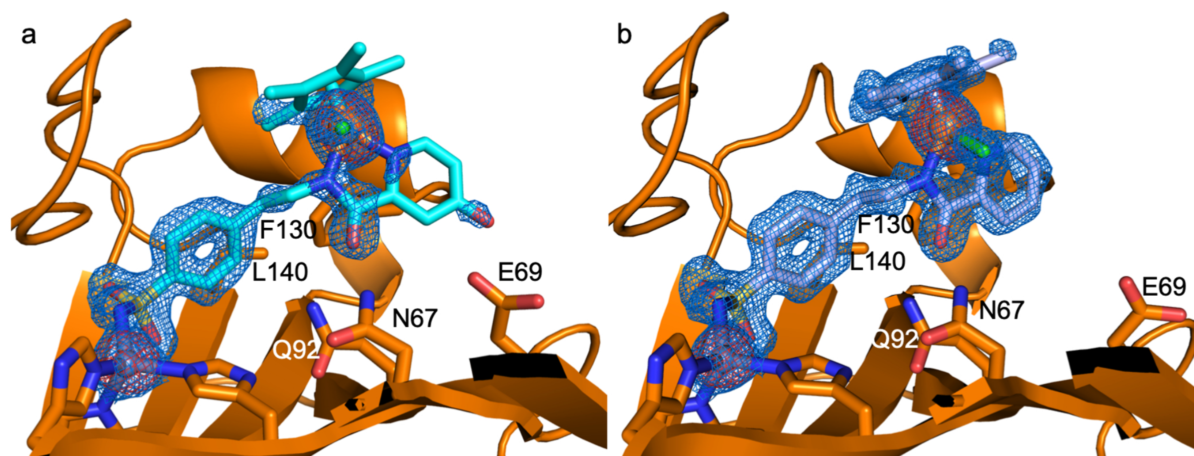


Figure S7. 2Fo-Fc electron density maps at 1.2 σ for cofactor **7** (a) and **8** (b) bound to CAII. Amino acids in the proximity of the cofactors are displayed as sticks and labelled; chloride, green, nitrogen, blue; oxygen, red; sulfur, yellow. The iridium and zinc ions are shown as spheres in orange and grey, respectively and surrounded by their respective anomalous electron densities (red mesh at 5 σ).

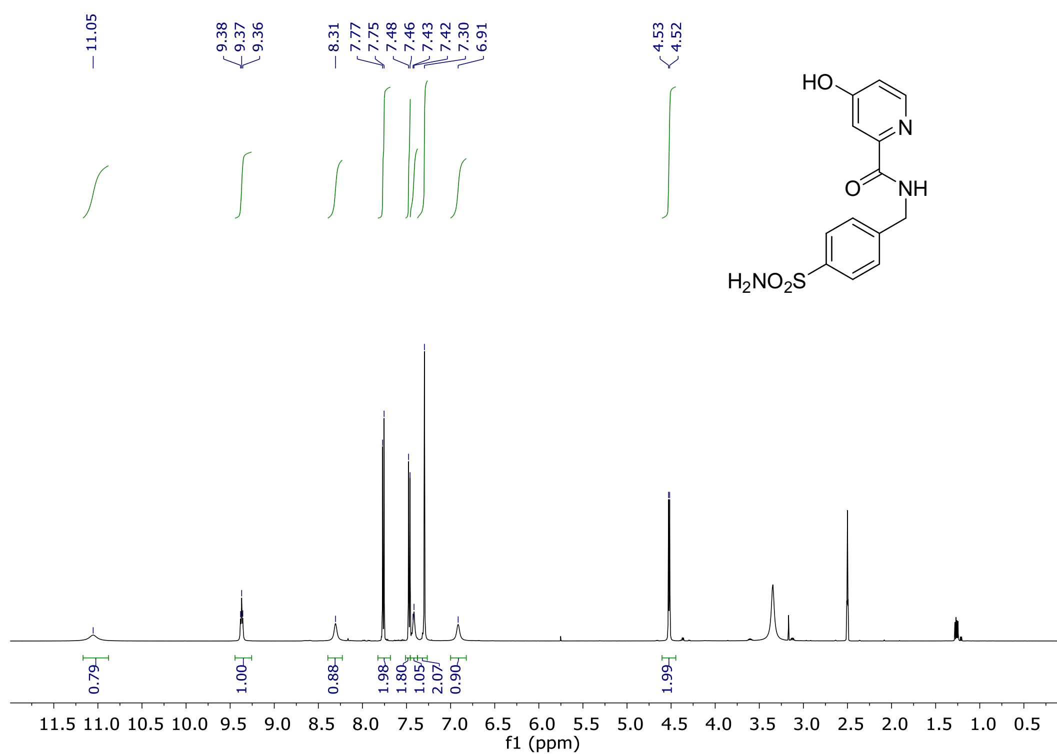


Figure S8. ^{1}H NMR spectrum of **11** in DMSO-d_6.

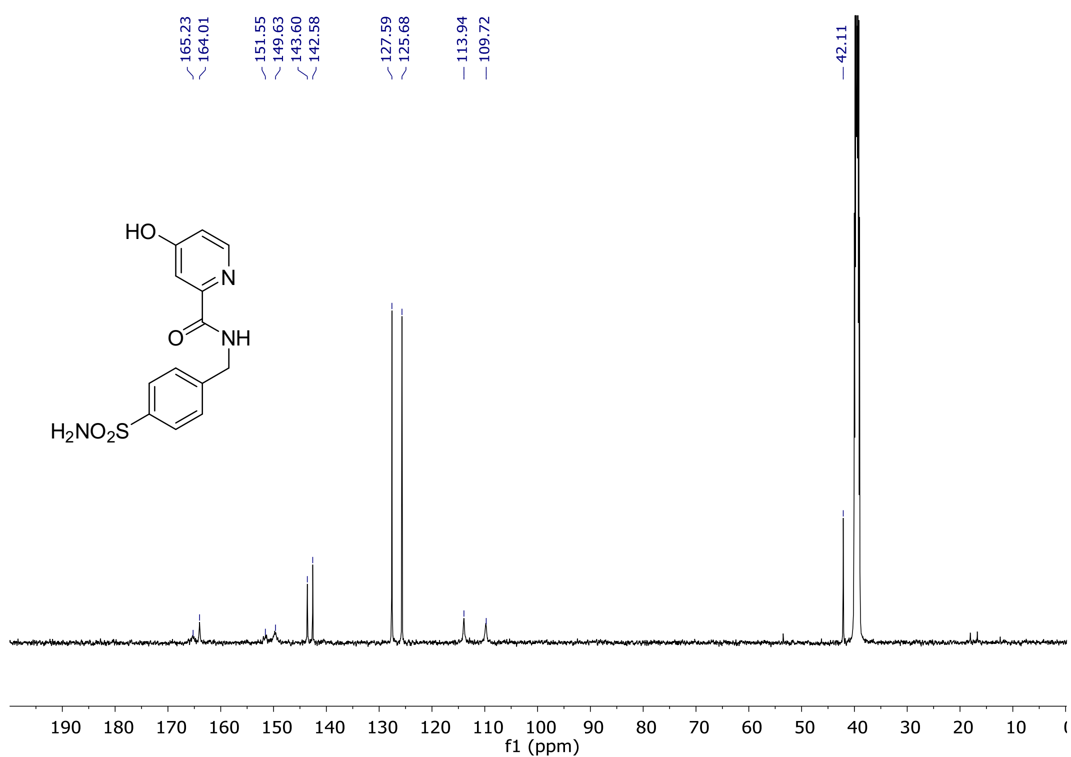


Figure S9. ^{13}C NMR spectrum of **11** in DMSO-d_6.

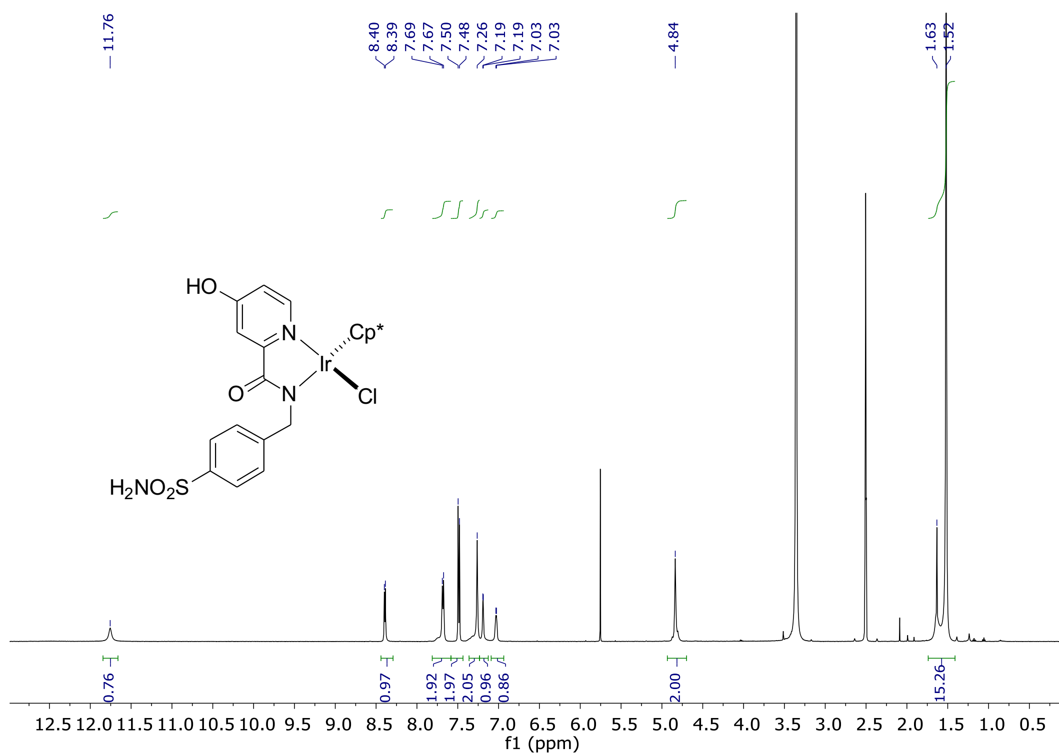


Figure S10. ^{1}H NMR spectrum of cofactor **6** in DMSO-d_6.

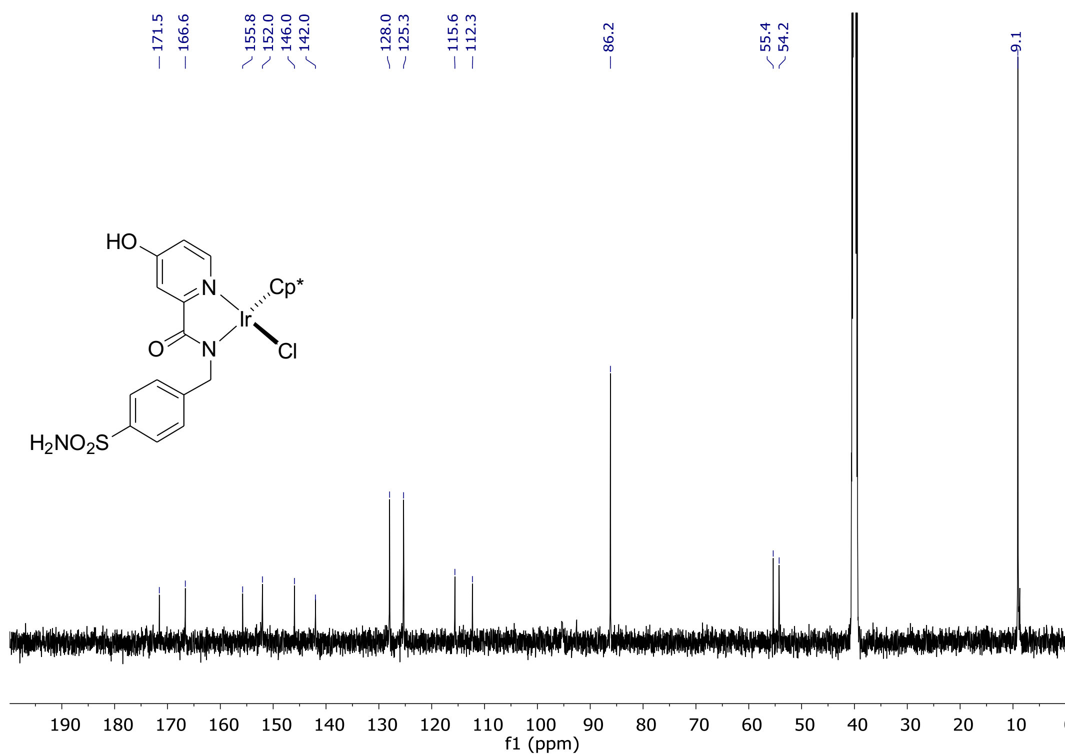


Figure S11. ^{13}C NMR spectrum of cofactor **6** in DMSO-d_6.

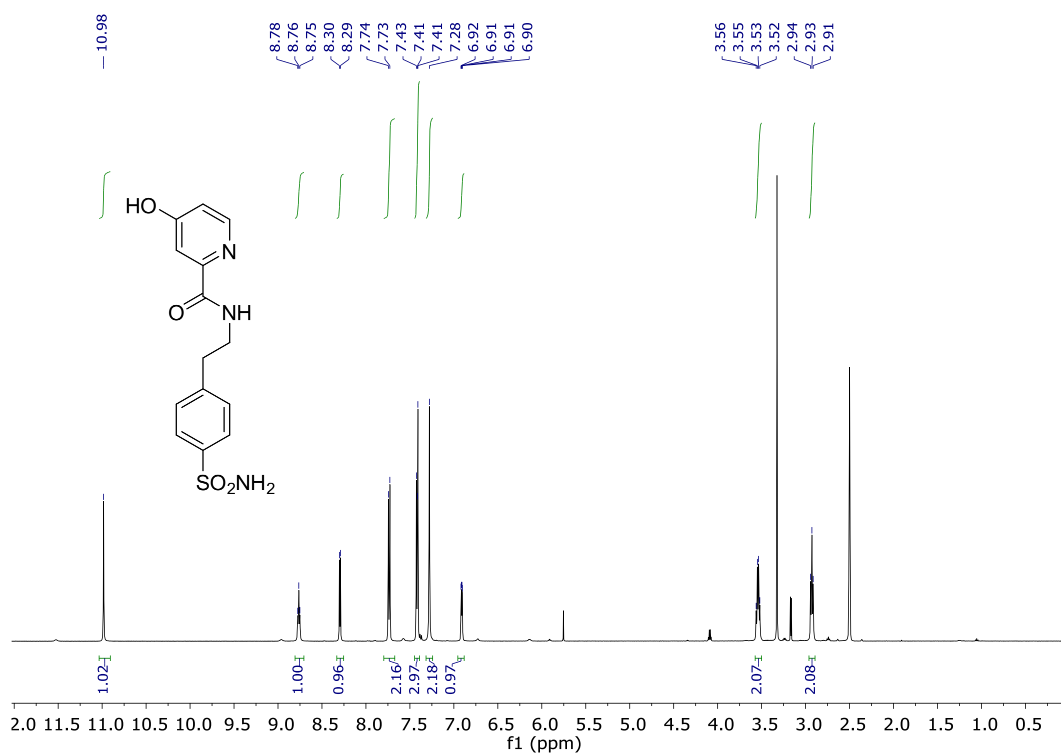


Figure S12. ^{1}H NMR spectrum of **12** in DMSO-*d6*.

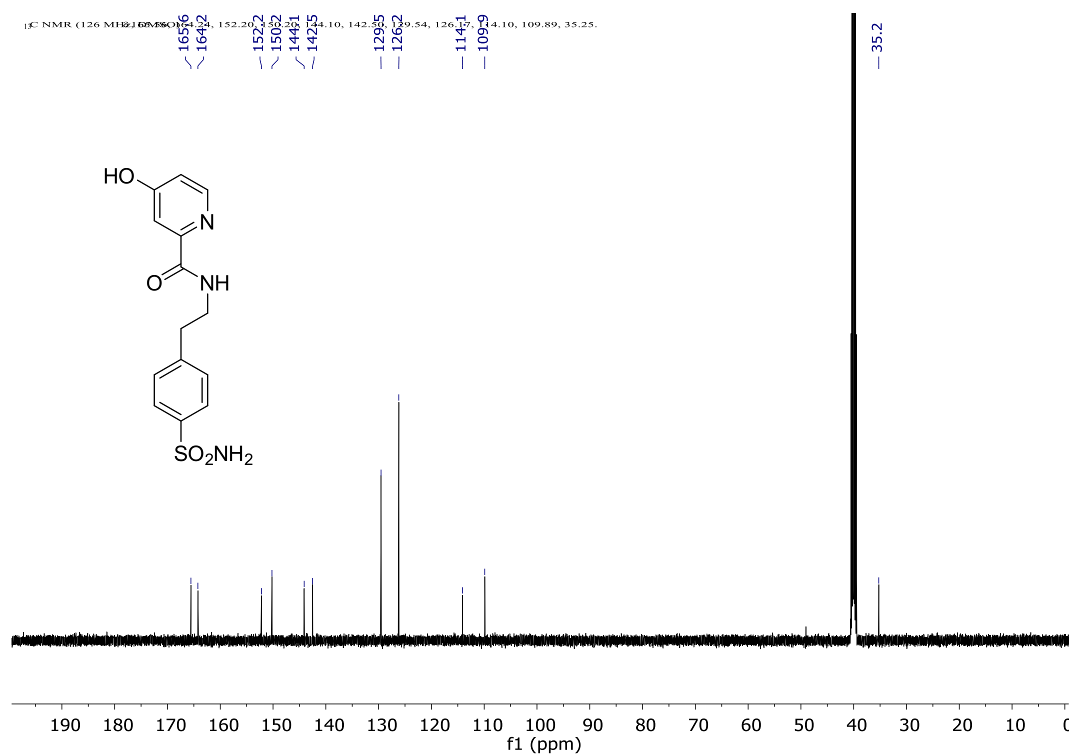


Figure S13. ^{13}C NMR spectrum of **12** in DMSO-*d6*.

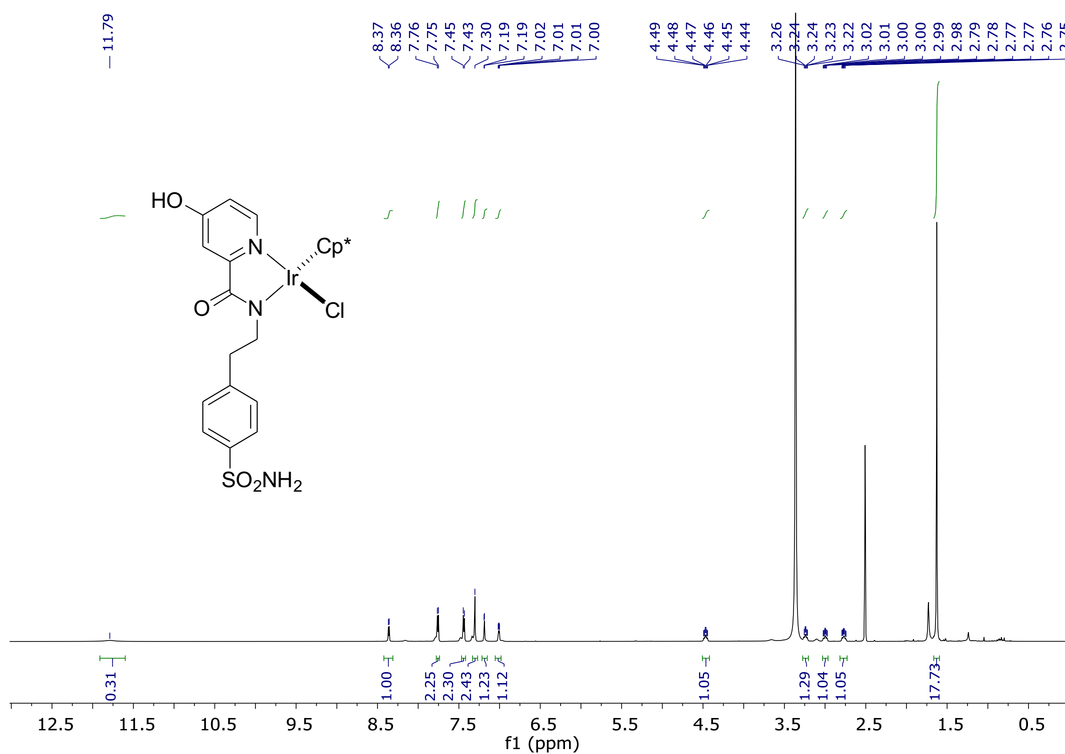


Figure S14. ^{1}H NMR spectrum of cofactor **7** in DMSO-d_6.

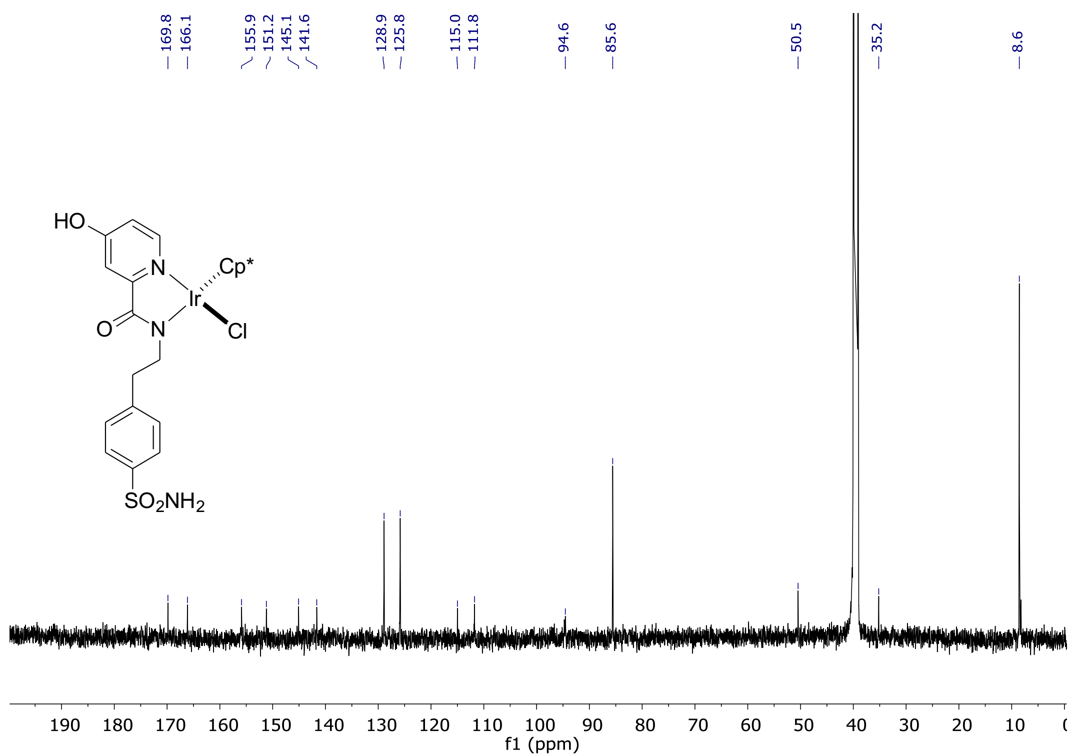


Figure S15. ^{13}C NMR spectrum of cofactor **7** in DMSO-d_6.

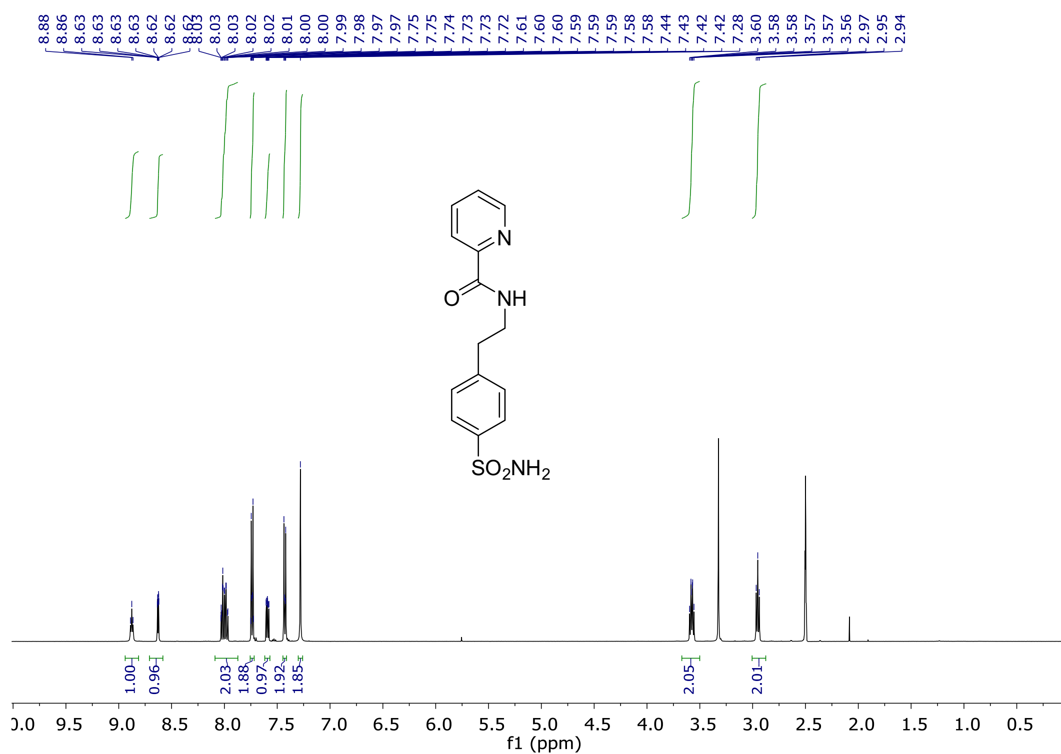


Figure S16. ^{1}H NMR spectrum of **13** in DMSO-*d*$_6$.

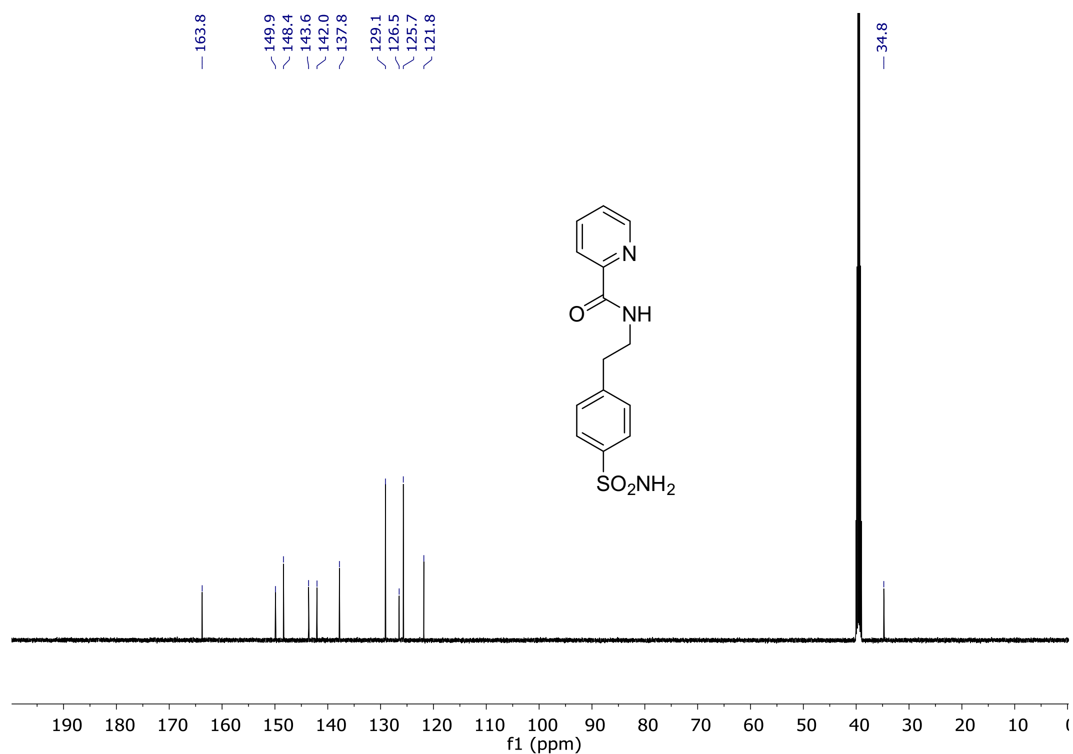


Figure S17. ^{13}C NMR spectrum of **13** in DMSO-*d*$_6$.

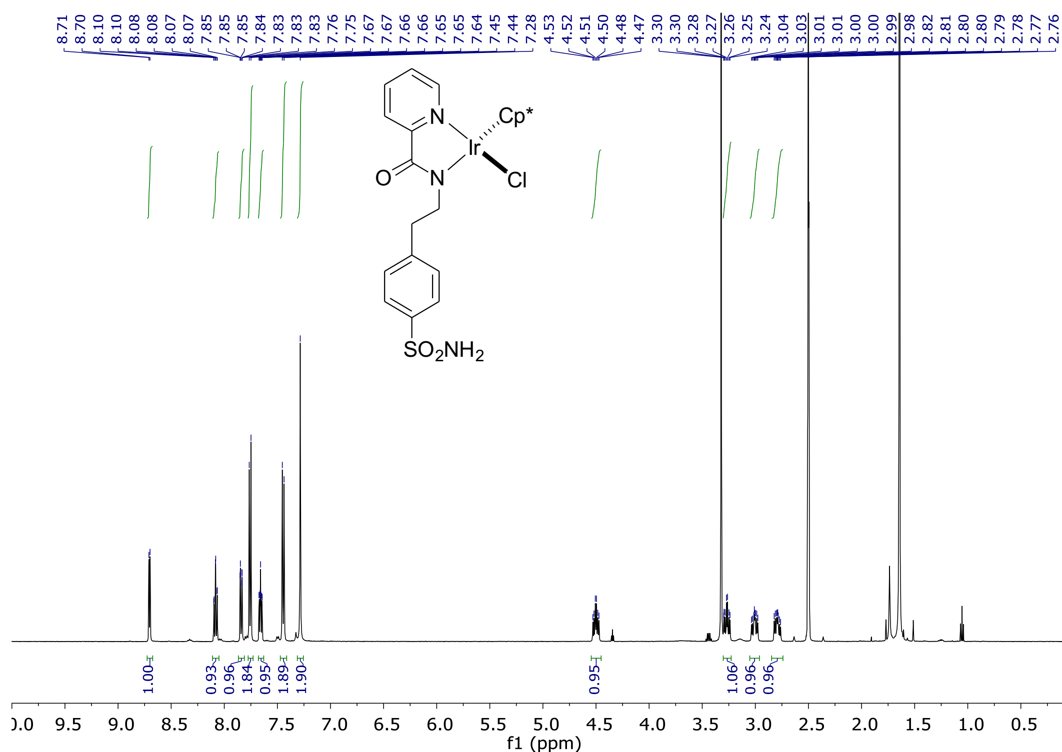


Figure S18. ^{1}H NMR spectrum of cofactor **8** in DMSO-*d*$_6$.

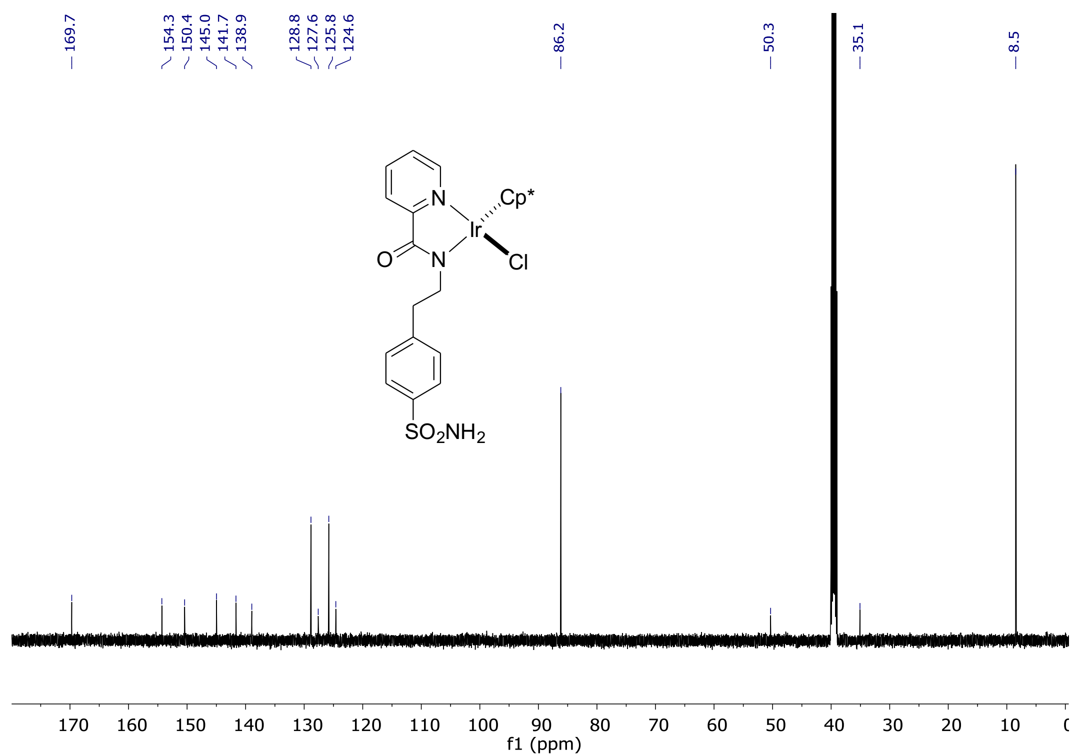


Figure S19. ^{13}C NMR spectrum of cofactor **8** in DMSO-*d*$_6$.

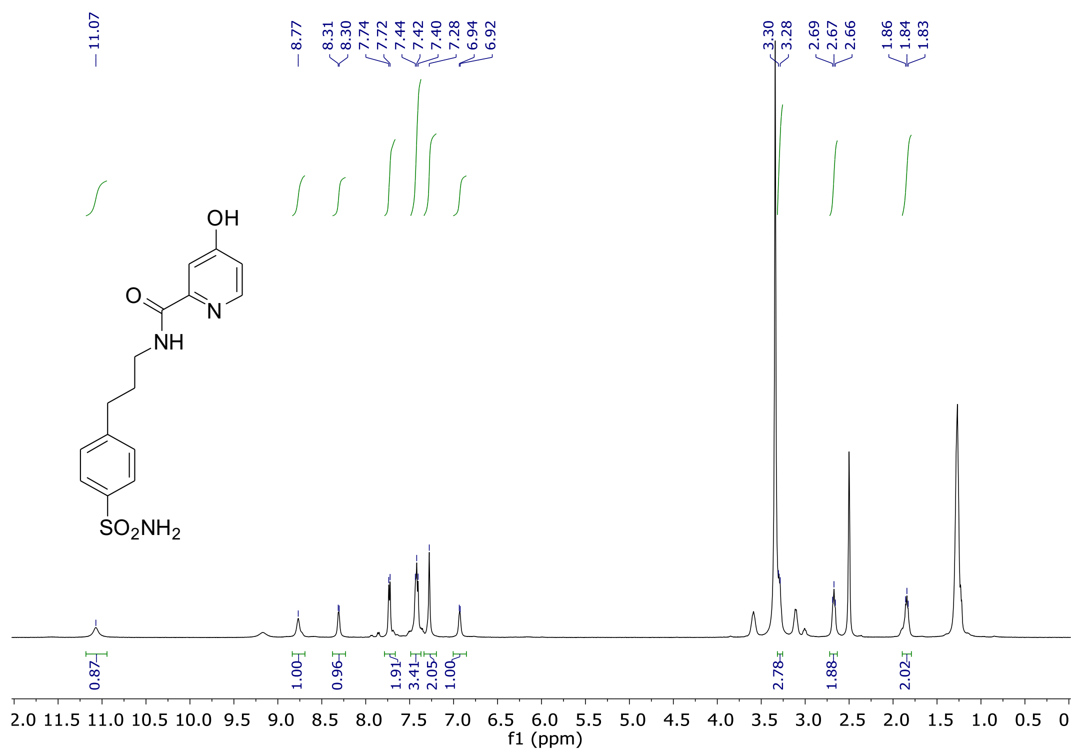


Figure S20. ^{1}H NMR spectrum of **15** in DMSO-d_6.

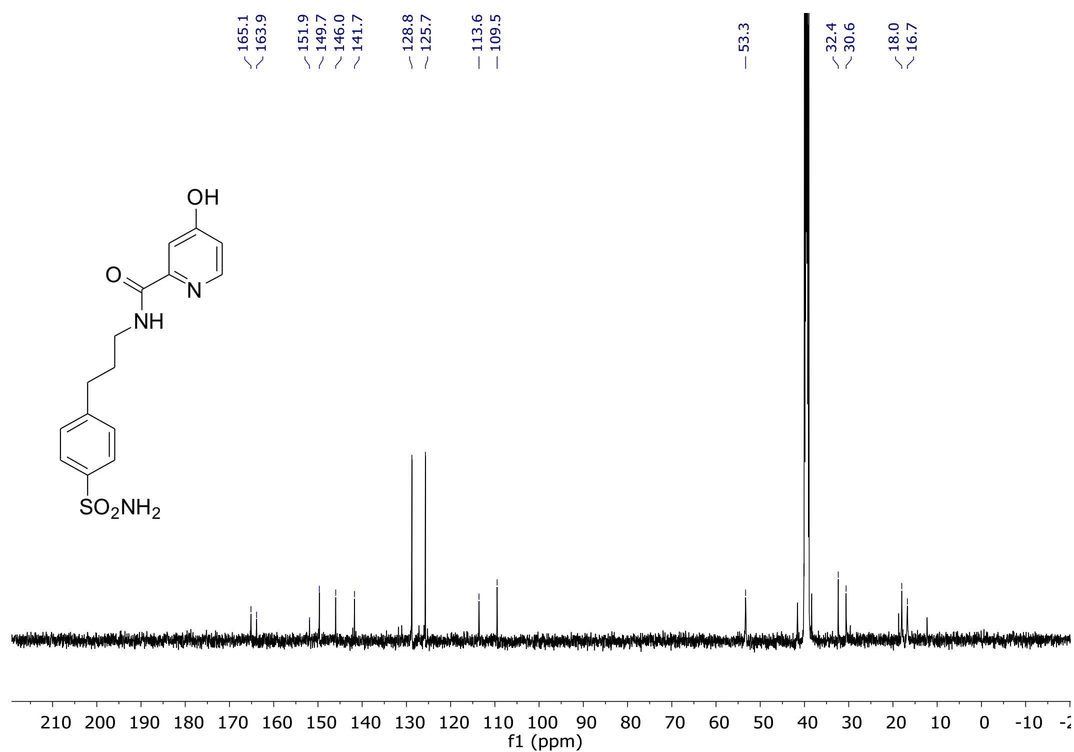


Figure S21. ^{13}C NMR spectrum of **15** in DMSO-d_6.

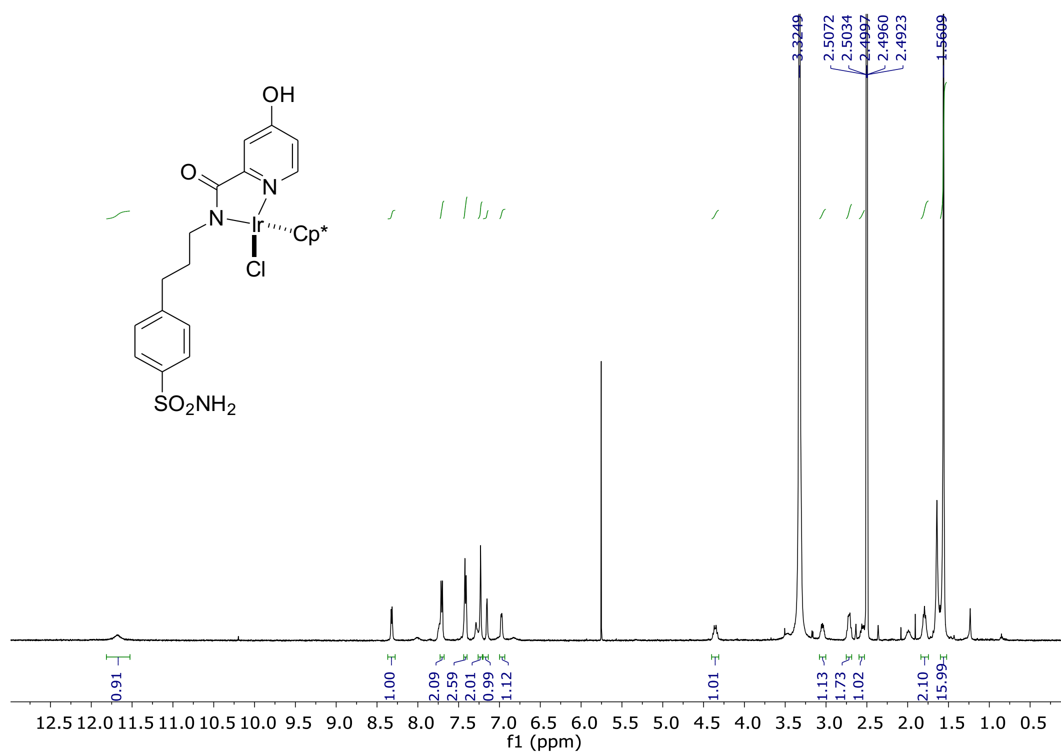


Figure S22. ^{1}H NMR spectrum of cofactor **9** in DMSO-*d*$_6$.

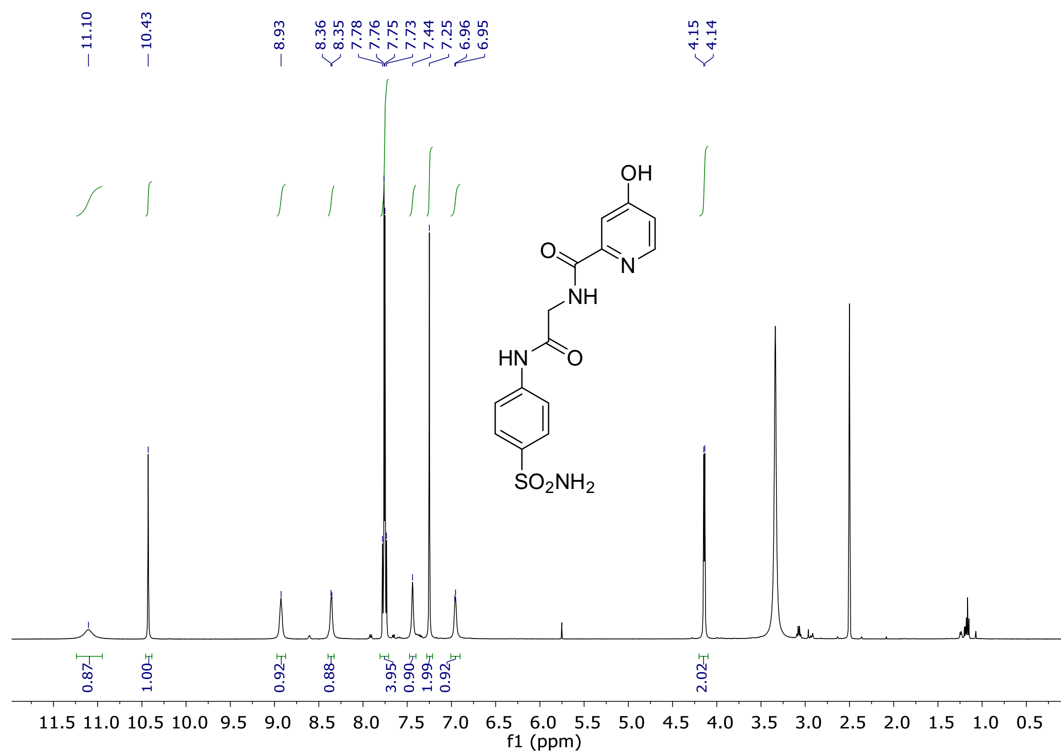


Figure S23. ^{1}H NMR spectrum of **17** in DMSO-*d*$_6$.

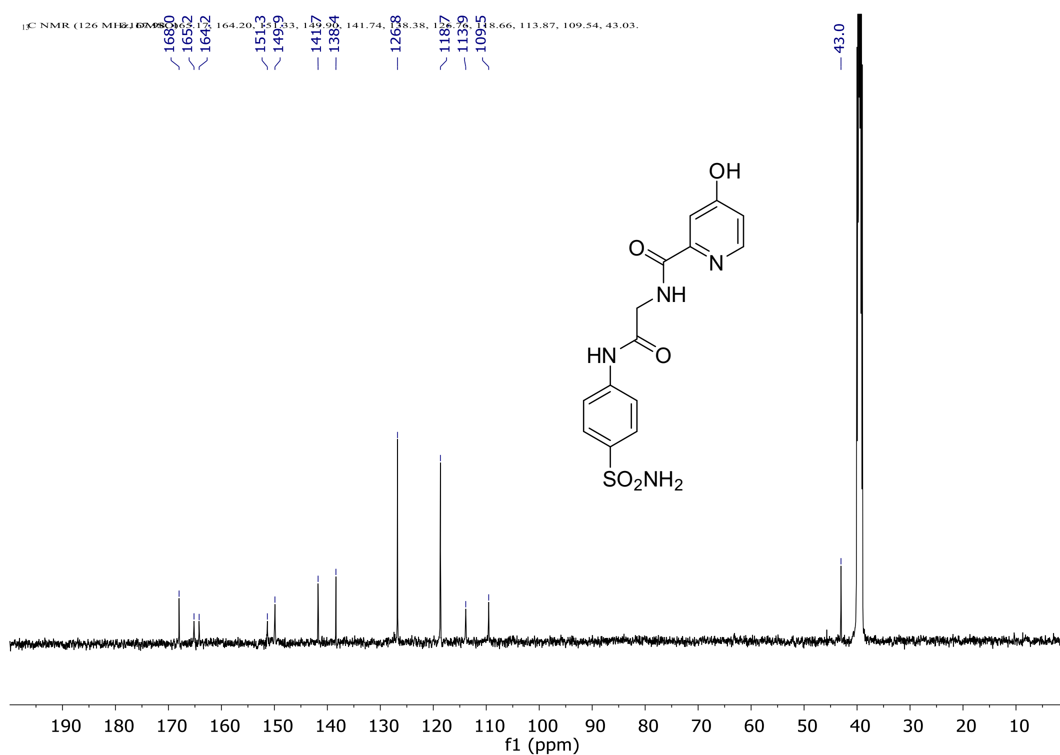


Figure S24. ^{13}C NMR spectrum of **17** in DMSO-*d*6.

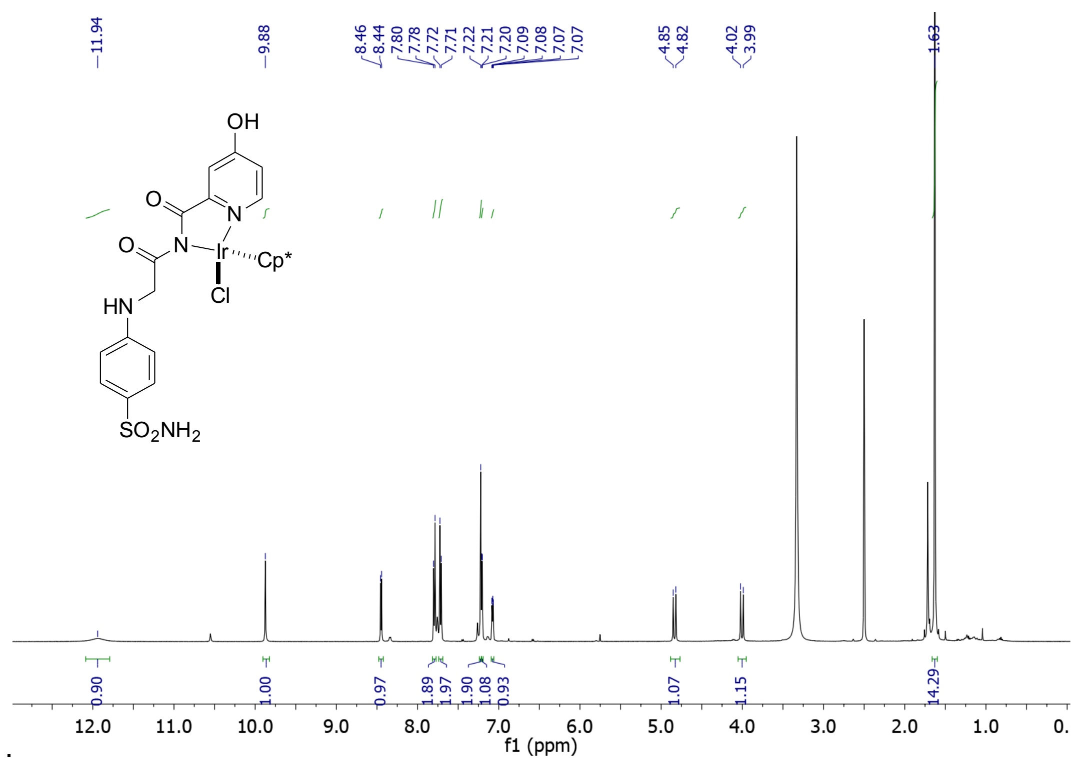


Figure S25. ^{1}H NMR spectrum of cofactor **10** in DMSO-*d*6.

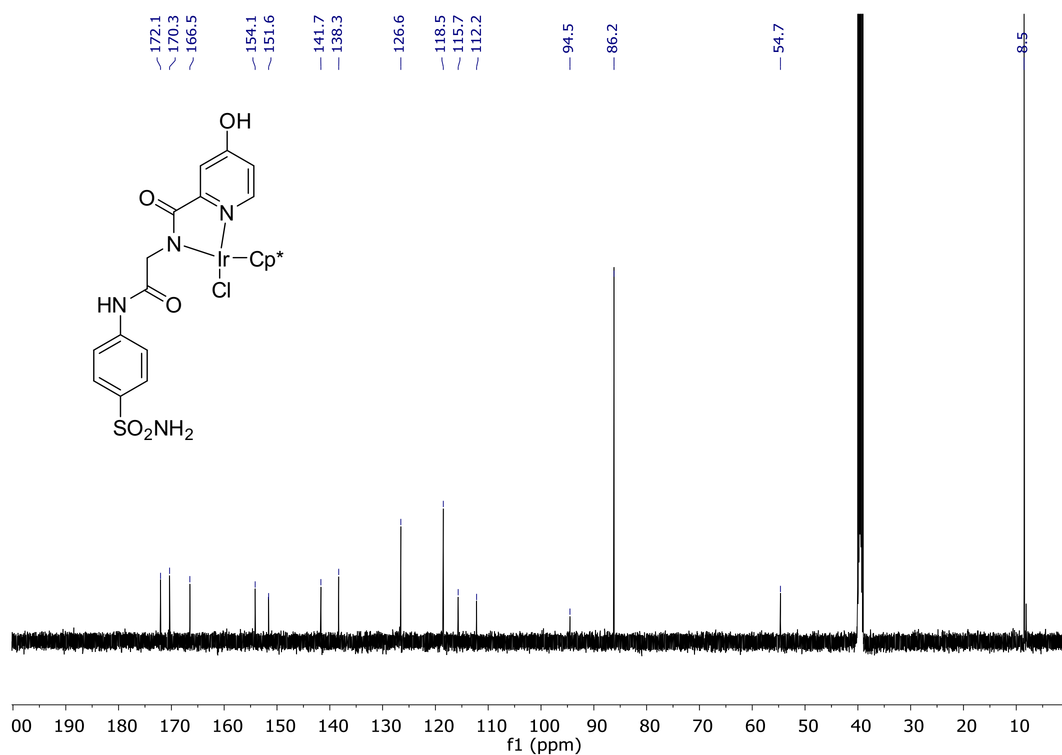


Figure S26. ^{13}C NMR spectrum of cofactor **10** in DMSO-d_6.

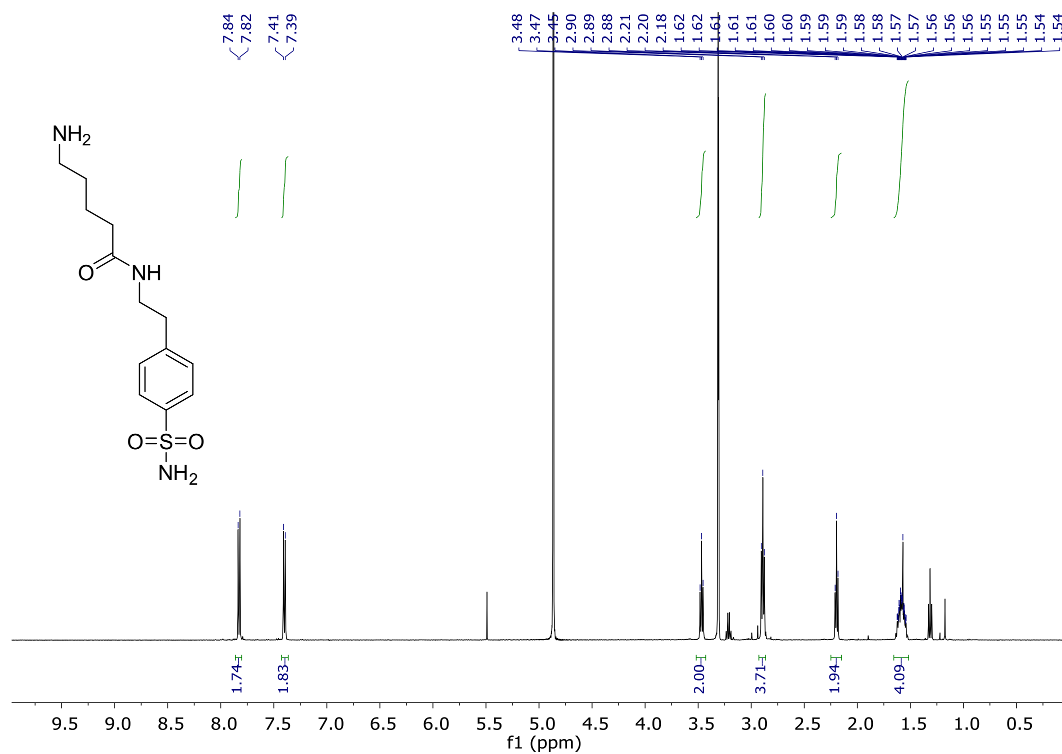


Figure S27. ^{1}H NMR spectrum of **18** in MeOD.

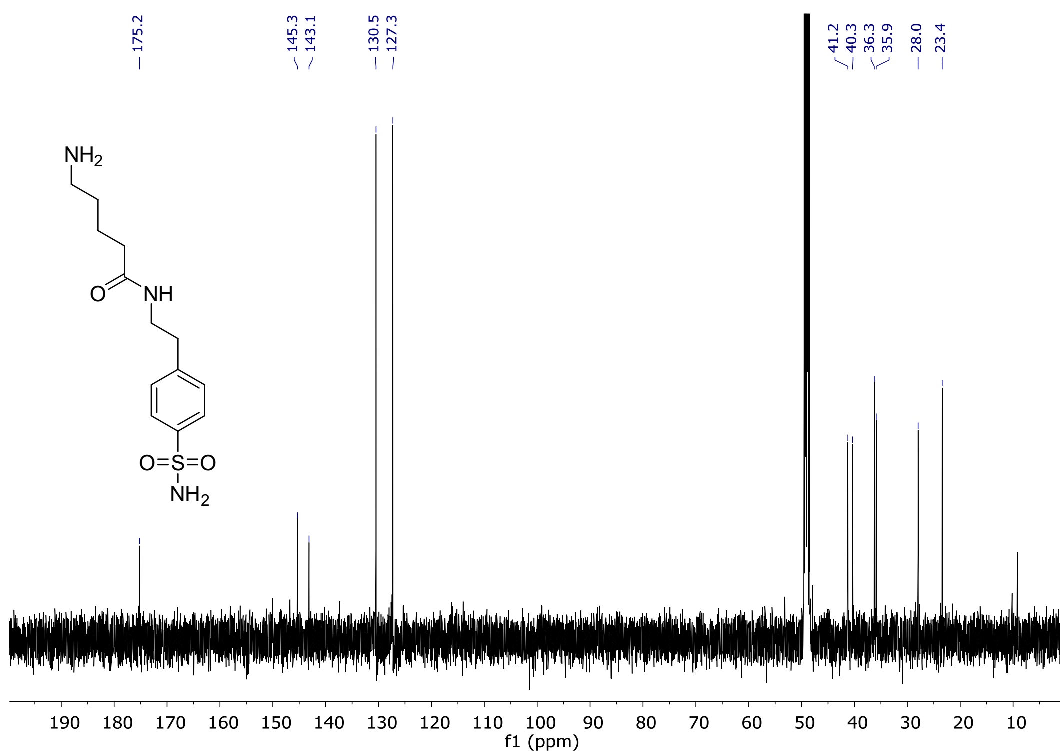


Figure S28. ^{13}C NMR spectrum of **18** in MeOD.

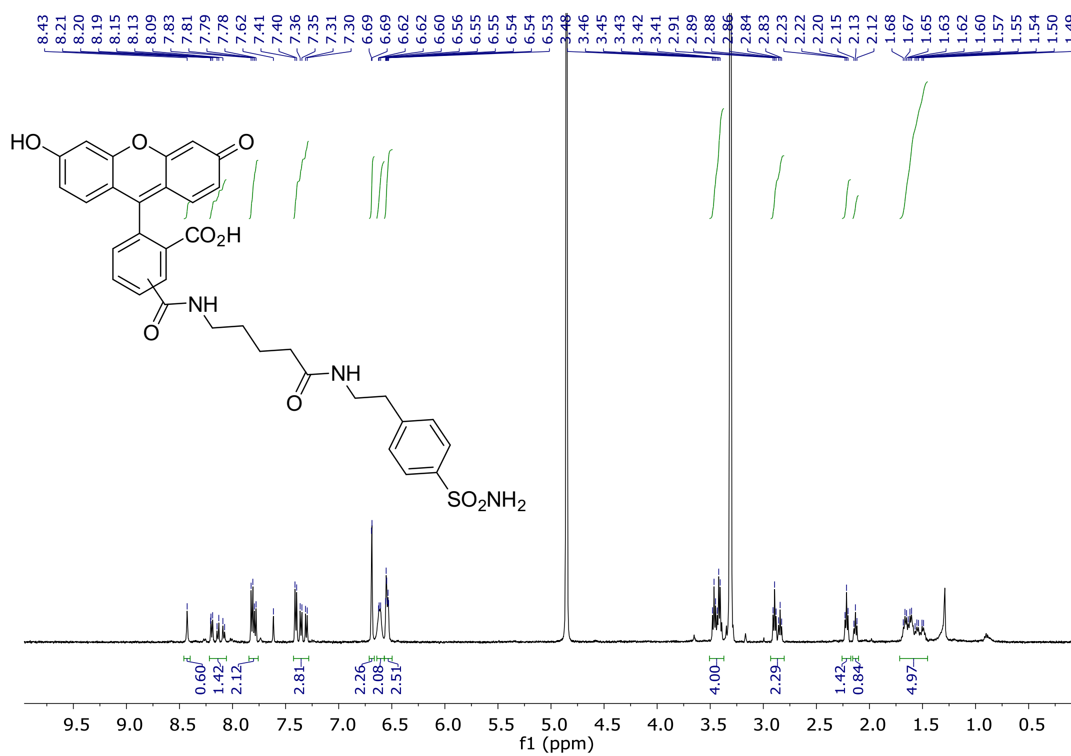


Figure S29. ^{1}H NMR spectrum of **4** in MeOD.

4. References

(1) Jeschek, M.; Reuter, R.; Heinisch, T.; Trindler, C.; Klehr, J.; Panke, S.; Ward, T. R., Directed evolution of artificial metalloenzymes for in vivo metathesis. *Nature* **2016,** *537*, 661-665.
(2) Heinisch, T.; Schwizer, F.; Garabedian, B.; Csibra, E.; Jeschek, M.; Vallapurackal, J.; Pinheiro, V. B.; Marliere, P.; Panke, S.; Ward, T. R., E. coli surface display of streptavidin for directed evolution of an allylic deallylase. *Chem. Sci.* **2018,** *9*, 5383-5388.
(3) Zhao, J.; Rebelein, J. G.; Mallin, H.; Trindler, C.; Pellizzoni, M. M.; Ward, T. R., Genetic Engineering of an Artificial Metalloenzyme for Transfer Hydrogenation of a Self-Immolative Substrate in Escherichia coli's Periplasm. *J. Am. Chem. Soc.* **2018,** *140*, 13171-13175.
(4) Monnard, F. W.; Nogueira, E. S.; Heinisch, T.; Schirmer, T.; Ward, T. R., Human carbonic anhydrase II as host protein for the creation of artificial metalloenzymes: the asymmetric transfer hydrogenation of imines. *Chem. Sci.* **2013,** *4*, 3269-3274.
(5) Manickam, M.; Pillaiyar, T.; Boggu, P.; Venkateswararao, E.; Jalani, H. B.; Kim, N. D.; Lee, S. K.; Jeon, J. S.; Kim, S. K.; Jung, S. H., Discovery of enantioselectivity of urea inhibitors of soluble epoxide hydrolase. *Eur. J. Med. Chem.* **2016,** *117*, 113-24.
(6) Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C. T., Carbonic anhydrase inhibitors. Synthesis of water-soluble, topically effective, intraocular pressure-lowering aromatic/heterocyclic sulfonamides containing cationic or anionic moieties: is the tail more important than the ring? *J. Med. Chem.* **1999,** *42*, 2641-50.
(7) Heinisch, T.; Pellizzoni, M.; Durrenberger, M.; Tinberg, C. E.; Kohler, V.; Klehr, J.; Haussinger, D.; Baker, D.; Ward, T. R., Improving the Catalytic Performance of an Artificial Metalloenzyme by Computational Design. *J. Am. Chem. Soc.* **2015,** *137*, 10414-10419.
(8) Zhao, J.; Kajetanowicz, A.; Ward, T. R., Carbonic anhydrase II as host protein for the creation of a biocompatible artificial metathesase. *Org. Biomol. Chem.* **2015,** *13*, 5652-5655.
(9) Monnard, F. W.; Heinisch, T.; Nogueira, E. S.; Schirmer, T.; Ward, T. R., Human carbonic anhydrase II as a host for piano-stool complexes bearing a sulfonamide anchor. *Chem. Commun.* **2011,** *47*, 8238-8240.
(10) Evans, P. R.; Murshudov, G. N., How good are my data and what is the resolution? *Acta Cryst. D* **2013,** *69*, 1204-14.
(11) Evans, P., Scaling and assessment of data quality. *Acta Crystallogr. D Biol. Crystallogr.* **2006,** *62*, 72-82.
(12) Kabsch, W., Xds. *Acta Crystallogr. D Biol. Crystallogr.* **2010,** *66*, 125-32.
(13) Winter, G.; Waterman, D. G.; Parkhurst, J. M.; Brewster, A. S.; Gildea, R. J.; Gerstel, M.; Fuentes-Montero, L.; Vollmar, M.; Michels-Clark, T.; Young, I. D.; Sauter, N. K.; Evans, G., DIALS: implementation and evaluation of a new integration package. *Acta Cryst. D* **2018,** *74*, 85-97.
(14) Winter, G., xia2: an expert system for macromolecular crystallography data reduction. *J. Appl. Crystallogr.* **2010,** *43*, 186-190.
(15) Potterton, L.; Agirre, J.; Ballard, C.; Cowtan, K.; Dodson, E.; Evans, P. R.; Jenkins, H. T.; Keegan, R.; Krissinel, E.; Stevenson, K.; Lebedev, A.; McNicholas, S. J.; Nicholls, R. A.; Noble, M.; Pannu, N. S.; Roth, C.; Sheldrick, G.; Skubak, P.; Turkenburg, J.; Uski, V.; von Delft, F.; Waterman, D.; Wilson, K.; Winn, M.; Wojdyr, M., CCP4i2: the new graphical user interface to the CCP4 program suite. *Acta Cryst. D* **2018,** *74*, 68-84.
(16) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S., Overview of the CCP4 suite and current developments. *Acta Cryst. D* **2011,** *67*, 235-42.
(17) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J., Phaser crystallographic software. *J. Appl. Crystallogr.* **2007,** *40*, 658-674.
(18) Murshudov, G. N.; Skubak, P.; Lebedev, A. A.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Long, F.; Vagin, A. A., REFMAC5 for the refinement of macromolecular crystal structures. *Acta Cryst. D* **2011,** *67*, 355-67.
(19) Kovalevskiy, O.; Nicholls, R. A.; Long, F.; Carlon, A.; Murshudov, G. N., Overview of refinement procedures within REFMAC5: utilizing data from different sources. *Acta Cryst. D* **2018,** *74*, 215-227.
(20) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K., Features and development of Coot. *Acta Cryst. D* **2010,** *66*, 486-501.
(21) Schwizer, F.; Okamoto, Y.; Heinisch, T.; Gu, Y.; Pellizzoni, M. M.; Lebrun, V.; Reuter, R.; Kohler, V.; Lewis, J. C.; Ward, T. R., Artificial Metalloenzymes: Reaction Scope and Optimization Strategies. *Chem. Rev.* **2018,** *118*, 142-231.