Supporting Information

Differentiating carrier protein interactions in biosynthetic pathways using dapoxyl solvatochromism

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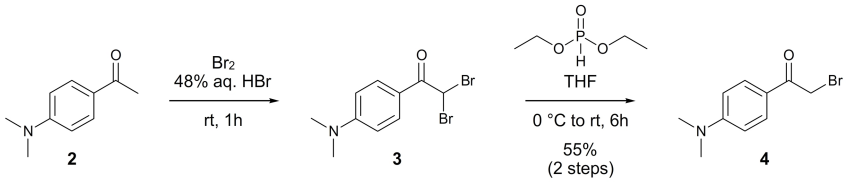
General synthetic methods. Chemical solvents and reagents were obtained from Acros Organics, Alfa Aesar, Chem-Impex International., Fisher Scientific, Sigma-Aldrich, or TCI Chemicals. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories. All reactions were conducted with rigorously dried solvents that were purchased from Alfa Aesar. Unless otherwise noted, all reactions were conducted in flame dried 100 mL, 50 mL, 1 dram or ½ dram vials equipped with a Teflon lined cap or septa and stir bar. Rt (rt) denotes 23±3 °C. Mixtures were heated on flask or reaction block adapters for IKAMAG RCT-basic stirrers (Chemglass). Analytical Thin Layer Chromatography (TLC) was performed on Silica Gel 60 F_{254} precoated glass plates (EM Sciences). Visualization was achieved with UV light and/or an appropriate stain ($KMnO_4$, dinitrophenylhydrazine, ninhydrin, and ceric ammonium molybdate). Flash chromatography was carried out on Fischer Scientific silica gel, 230-400 mesh, grade 60. Yields correspond to isolated, chromatographically, and spectroscopically homogeneous materials. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVA300, a JEOL400, or a Varian VX500 (equipped with an XSens Cold probe) spectrometer. ^{13}C-NMR spectra were recorded with proton decoupling. FID files were processed using Mnova 14.2.3 (MestreLab Research). A Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer was used for high-resolution electrospray ionization mass spectrometry analysis (HR-ESI-MS). Procedures are provided for all new compounds and copies of NMR spectra have been provided within the Supporting Information.

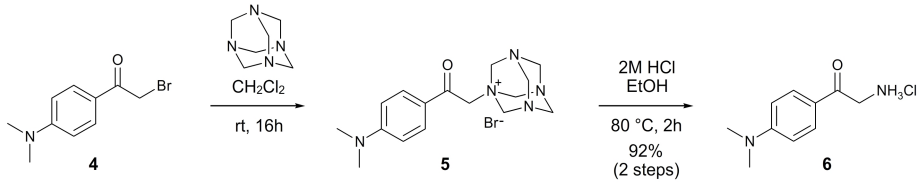
2-Bromo-1-(4-(dimethylamino)phenyl)ethan-1-one (4).



Preparation of **4** was best conducted in a single protocol. 1-(4-(Dimethylamino)phenyl)ethan-1-one (**2**, 2.00 g, 12.3 mmol) was dissolved 48% w/w aq. HBr (9 mL). Bromine (2.35 g, 14.7 mmol) dissolved in 1 mL 48% w/w aq. HBr (1 mL) was added dropwise over 10 min. The reaction was stirred for 1 h at rt and poured into an satd. $NaHCO_3$ solution (50 mL). Solid $NaHCO_3$ was added to fully neutralize the solution. The aq. solution was extracted with CH_2Cl_2 (3 × 30 mL). The organic layers were combined, washed with brine (30 mL), dried with $MgSO_4$, filtered through a pad of cotton, and concentrated under vacuum. The remaining yellow solid was dissolved in THF (25 mL) and cooled to 0 °C. Diethyl phosphonate (1.69 g, 12.3 mmol) was added dropwise over 5 min. Triethylamine (1.24 g, 12.3 mmol) was added dropwise over 5 min. The flask was warmed to rt and stirred for 6 h. The solvent was evaporated to an approximate volume of 5 mL and was poured into H_2O (50 mL). The crude material was obtained by vacuum filtration. Pure bromide **4** (1.62 g, 55%) was obtained by crystallization from EtOH as light-yellow crystals.

Bromide **4**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 9.2 Hz, 2H), 6.69 (d, J = 9.0 Hz, 2H), 4.37 (s, 2H), 3.09 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.55, 153.88, 131.46, 111.01, 40.24, 30.89. HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{10}H_{13}BrNO$ $[M+H]^+$: 242.0175, found 242.0173.

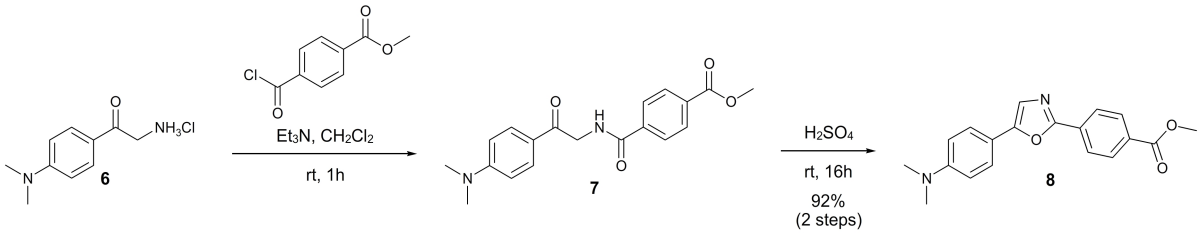
2-Amino-1-(4-(dimethylamino)phenyl)ethan-1-one dihydrochloride (6).



This conversion of **4** to **6** was best conducted in a two-step single protocol. Hexamethylenetetramine (290 mg, 2.07 mmol) was added to a solution of **4** (334 mg, 1.38 mmol) in $CHCl_3$ (14 mL). The reaction was stirred overnight at rt. The quaternary ammonium salt intermediate **5**, which precipitated from solution, was obtained by vacuum filtration. The solid was redissolved in 2 M HCl in 2:1 EtOH/H_2O (15 mL) and stirred at reflux for 1 h. The solvent was evaporated, leaving the crude product as a hydrochloride salt. Pure **6** (207 mg, 60%) was obtained by crystallization from EtOH as an off-white crystalline solid.

Ammonium salt **6**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.15 (s, 3H), 7.83 (d, J = 9.2 Hz, 2H), 6.76 (d, J = 9.2 Hz, 2H), 4.41 (d, J = 5.0 Hz, 2H), 3.05 (s, 6H). Poor solubility prevented ^{13}C NMR analysis. HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{10}H_{15}N_2O$ $[M+H]^+$: 179.1179, found 179.1178.

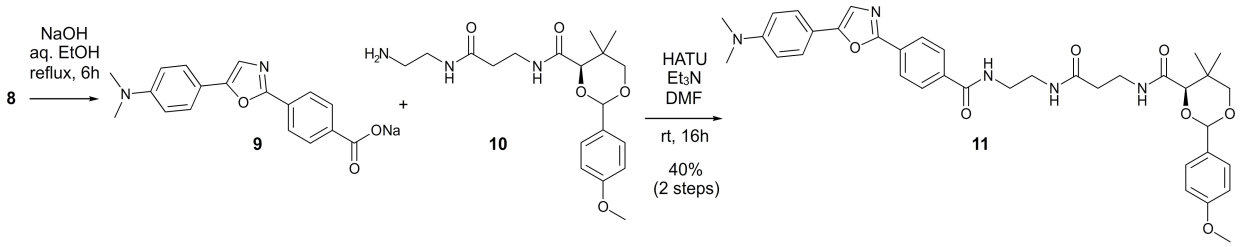
Methyl 4-(5-(4-(dimethylamino)phenyl)oxazol-2-yl)benzoate (8).



A two-step protocol was used to convert **6** to **8**. Ammonium salt **6** (120 mg, 0.478 mmol), methyl 4-(chlorocarbonyl)benzoate (104 mg, 0.526 mmol), and triethylamine (145 mg, 1.43 mmol) were added to dry CH_2Cl_2 (5 mL). The solution was stirred for 1 h at rt. The solution was diluted with additional CH_2Cl_2 (15 mL) and washed sequentially with satd. $NaHCO_3$ (20 mL) and satd. NH_4Cl (20 mL). The organic layer was dried with $MgSO_4$, filtered through cotton, and evaporated under vacuum. The crude intermediate **7** was further purified from organic impurities by resuspension in EtOAc, heating to reflux, and vacuum filtration. The insoluble portion was redissolved in concentrated H_2SO_4 (5 mL) and stirred overnight at rt. The solution was neutralized by addition to satd. $NaHCO_3$ (20 mL), immediately resulting in formation of a bright yellow solid. The resulting suspension was vacuum filtered and pure **8** (142 mg, 92%) was obtained as bright yellow solid.

Intermediate **8**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13 (s, 4H), 7.60 (d, J = 9.0 Hz, 2H), 7.30 (s, 1H), 6.76 (d, J = 8.8 Hz, 2H), 3.94 (s, 3H), 3.02 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.73, 158.96, 153.06, 150.61, 131.61, 130.84, 130.18, 125.83, 125.77, 121.22, 115.73, 112.28, 52.45, 40.47, 29.84. HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{19}H_{19}N_2O_3$ $[M+H]^+$: 323.1390, found 323.1393.

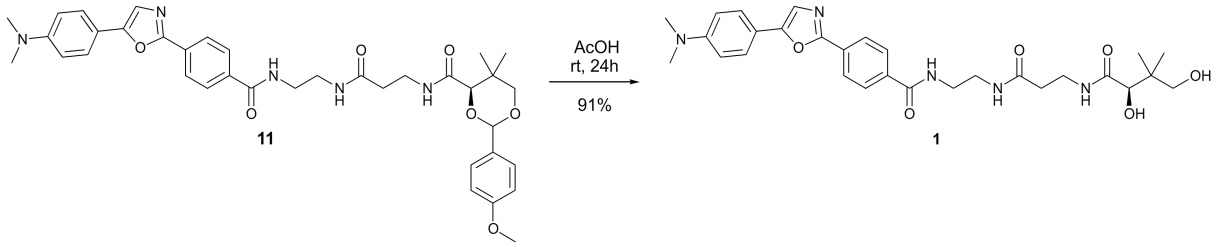
(4*R*)-*N*-(3-((2-(4-(5-(4-(dimethylamino)phenyl)oxazol-2-yl)benzamido)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (11).



A two-step procedure was again beneficial to improve the efficiency in the production of **11**. Methyl ester **8** (44 mg, 0.14 mmol) was dissolved in EtOH (7 mL), to which 2 M aq. NaOH (7 mL) was added. The solution was heated to reflux and stirred for 6 h. The reaction was neutralized by addition of 6 M aq. HCl (1-2 mL), resulting in formation of an orange precipitate. The solvent was evaporated under vacuum and the precipitate redissolved in dry DMF (14 mL). HATU (100 mg, 0.27 mmol) was added, followed by **10**[1] (78 mg, 0.20 mmol) and triethylamine (41 mg, 0.41 mmol). The reaction was stirred at rt overnight. H_2O (20 mL) was added to quench the reaction, which was subsequently extracted with EtOAc (3 × 20 mL). The organic solution was sequentially washed with satd. $NaHCO_3$ (20 mL), satd. NH_4Cl (20 mL), brine (20 mL), dried with $MgSO_4$, filtered through cotton, and concentrated under vacuum. Protected probe **11** (37 mg, 40%) was obtained as a bright yellow solid via silica flash column chromatography, eluting with a gradient of 1:19 to 1:9 MeOH/CH_2Cl_2.

Protected probe **11**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (d, *J* = 8.5 Hz, 2H), 7.92 (d, *J* = 8.6 Hz, 2H), 7.62 (d, *J* = 8.4 Hz, 2H), 7.50 (s, 1H), 7.40 (d, J = 8.7 Hz, 2H), 7.30 (s, 1H), 7.01 (t, *J* = 6.4 Hz, 1H), 6.91 (s, 1H), 6.88 (d, *J* = 8.8 Hz, 2H), 5.43 (s, 1H), 4.07 (s, 1H), 3.77 (s, 3H), 3.73 – 3.60 (m, 2H), 3.60 – 3.38 (m, 6H), 3.04 (s, 6H), 2.48 (t, *J* = 6.2 Hz, 2H), 1.08 (d, *J* = 10.9 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.54, 170.12, 167.32, 160.34, 134.83, 130.38, 130.10, 129.94, 127.74, 127.67, 126.15, 125.78, 113.82, 101.47, 83.87, 78.48, 55.44, 43.73, 41.42, 39.83, 36.79, 35.02, 33.24, 21.97, 19.23. HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{33}H_{44}N_5O_7$ $[M+H]^+$: 670.3235, found 670.3236.

(*R*)-*N*-(2-(3-(2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)-4-(5-(4-(dimethylamino)phenyl)oxazol-2-yl)benzamide (1)



PMB acetal-protected adduct **11** (36 mg, 54 μmol) was dissolved in a 4:1 mixture of acetic acid/H_2O and stirred at rt overnight. The solution was concentrated under vacuum. Probe **1** (27 mg, 91%) was obtained as a yellow solid via flash column chromatography, eluting with a gradient of 1:9 to 1:4 MeOH/CH_2Cl_2.

Probe **1**: ^{1}H NMR (500 MHz, CD_3OD) δ 8.11 (d, *J* = 8.5 Hz, 2H), 7.97 (d, *J* = 8.5 Hz, 2H), 7.63 (d, *J* = 8.9 Hz, 2H), 7.39 (s, 1H), 6.82 (d, *J* = 8.9 Hz, 2H), 3.89 (s, 1H), 3.55 – 3.38 (m, 8H), 3.01 (s, 6H), 2.45 (t, *J* = 6.6 Hz, 2H), 0.92 (d, *J* = 1.8 Hz, 6H). ^{13}C NMR (126 MHz, CD_3OD) δ 176.14, 174.40, 169.44, 160.20, 154.81, 152.37, 136.69, 131.08, 129.14, 126.84, 126.64, 121.24, 116.52, 113.39, 77.17, 70.22, 40.87, 40.43, 40.36, 40.07, 36.64, 36.44, 21.37, 20.84. HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{29}H_{37}N_5O_6$ $[M+Na]^+$: 574.2636, found 574.2639.

Protein expression and purification. Polyhistidine-tagged proteins AcpP,[2] hACP,[1] DEBS Acp4,[3] actACP C17S,[4] ΔEntB,[5] PltL,[6] FabF,[7] FabB,[7] FabF,[7] FabH,[7] FabG,[8] FabA,[9] FabI,[10] FabD,[11] CoaA,[12] CoaD,[12] CoaE,[12] Sfp,[12] and AcpH[2] were expressed and purified based on previously described methods. A general procedure for expression and purification is described herein. A plasmid with a gene encoding the protein of interest controlled by a T7 *lac*-inducible promoter was transformed into BL21 (DE3) competent cells, which were selected on 100 mg/L ampicillin or 50 mg/L kanamycin-containing LB agar. Single colonies were grown in LB/ampicillin or LB/kanamycin at 37 °C to OD_{600} = 0.6-0.8 prior to induction with 1 mM IPTG at 16 °C for 16 hours. Cells were harvested by centrifugation at 2000 RPM for 40 min and separated into cell pellets (5-10 g). Pellets were resuspended in TBS (50 mM Tris, 150 mM NaCl, optional 10% glycerol v/v, pH = 7.4) and lysed by sonication. Cell lysate was spun at 4700 RPM for 1 h to pellet cell debris. The clarified supernatant was filtered through a Kimwipe and applied to a pre-equilibrated column containing 1 mL of Ni-NTA resin. The protein-loaded resin was washed sequentially with TBS (50 mL) and 10 mM imidazole in TBS (50 mL). The protein was eluted with 250 mM imidazole in lysis buffer and dialyzed overnight at 4 °C against TBS (no glycerol). Proteins were concentrated to 2-10 mg/mL using Amicon® Ultra-15 centrifugal filter units and stored in flash frozen aliquots at −80 °C. Concentrations were determined using NanoDrop spectrophotometeric and/or Bradford analyses. Purity was confirmed to be greater than 90% by LC-MS and/or SDS-PAGE analyses.

Preparation of *apo*-AcpP. AcpP expressed as a mixture of 4'-phosphopantetheinylated *holo*- and unmodified *apo*- forms and required extra steps to be fully converted to its *apo*- form. Following Ni-NTA elution, AcpP was incubated with AcpH (0.01 mg/mL) and simultaneously dialyzed against 50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, pH 7.2 buffer overnight at 4 °C. The solution was passed through a sterile 0.2 μM filter and applied to a 5 mL HiTrap Q HP anion exchange column (Cytiva). The protein was eluted over a linear gradient of 50 mM Tris, pH 7.4 buffer containing 0 to 1 M NaCl (20 CV). Fractions containing *apo*-AcpP were identified by SDS-PAGE, pooled, and dialyzed overnight (16 h) at 4 °C against 50 mM Tris, 150 mM NaCl, pH 7.4 buffer. *apo*-AcpP was concentrated and stored in flash frozen aliquots at −80 °C.

Dapoxyl-CP probe loading. In the following order, reactants were mixed in TBS (50 mM Tris, 150 mM NaCl, pH = 7.4): 1 mM DTT, 8 mM ATP, 12.5 mM $MgCl_2$, 0.01 mg/mL CoaA, 0.01 mg/mL CoaD, 0.01 mg/mL CoaE, 0.04 mg/mL Sfp, 0.5 mM Dapoxyl-pantetheinamide, and 0.1 mM *apo*-CP. The mixture was incubated overnight (16 h) at ambient temperature. Conversion of *apo*-CP to probe-loaded *crypto*-CP was analyzed by LC-MS, HPLC, and/or confirmation-sensitive urea-PAGE. Prior to protein fixation and Coomassie Blue staining, urea-PAGE gels were additionally visualized under UV light (365 nm).

Fluorescence studies. Fluorescent samples (30 μL) were pipetted into a Greiner microplate (96 well, PS, half area, black, med. binding) in triplicate. Fluorescent emission spectra were collected using a ThermoFisher VarioLux plate reader within 30 minutes of reaction setup. The following parameters were used (unless otherwise stated): excitation wavelength, 370 nm; emission wavelength range, 390-800 nm; measurement time, 100 ms.

Partner enzyme and inhibitor studies. The following reagents were mixed together in TBS (50 mM Tris, 150 mM NaCl, pH = 7.4): Dapoxyl-AcpP (50 μM, directly from probe loading reaction without purification), partner protein (50 μM), and cerulenin (1-100 μM, only for inhibitor studies). Fluorescence measurements were taken as detailed under "Fluorescence studies". For

inhibitor studies, emission maxima from each spectrum were extracted and plotted versus concentration of cerulenin. The points were fitted to a general Hill ligand-binding equation: $F = \frac{a[L]^n}{IC_{50}^n + [L]^n} + b$. Parameters for a (scaling factor), b (minimum emission), IC_{50} value (apparent dissociation constant), and n (Hill coefficient) were calculated using least squares fitting to the Hill equation.

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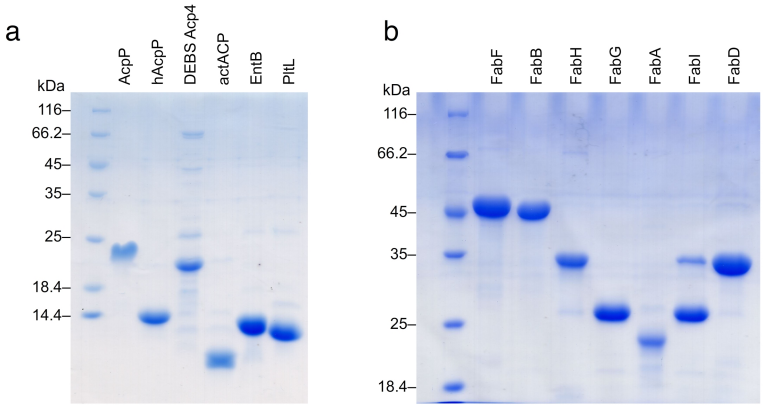
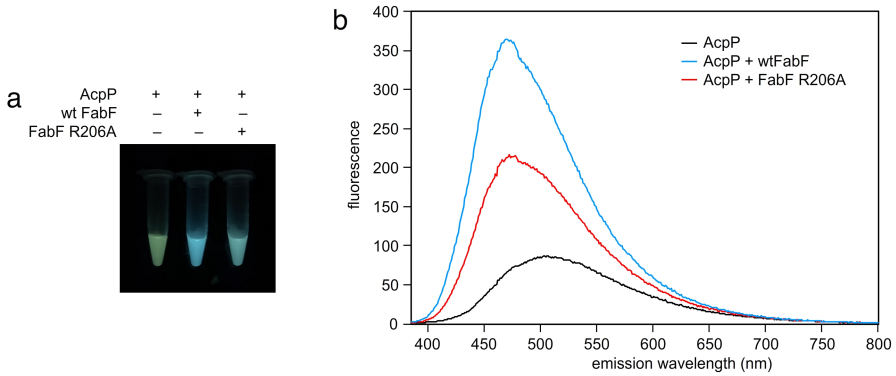
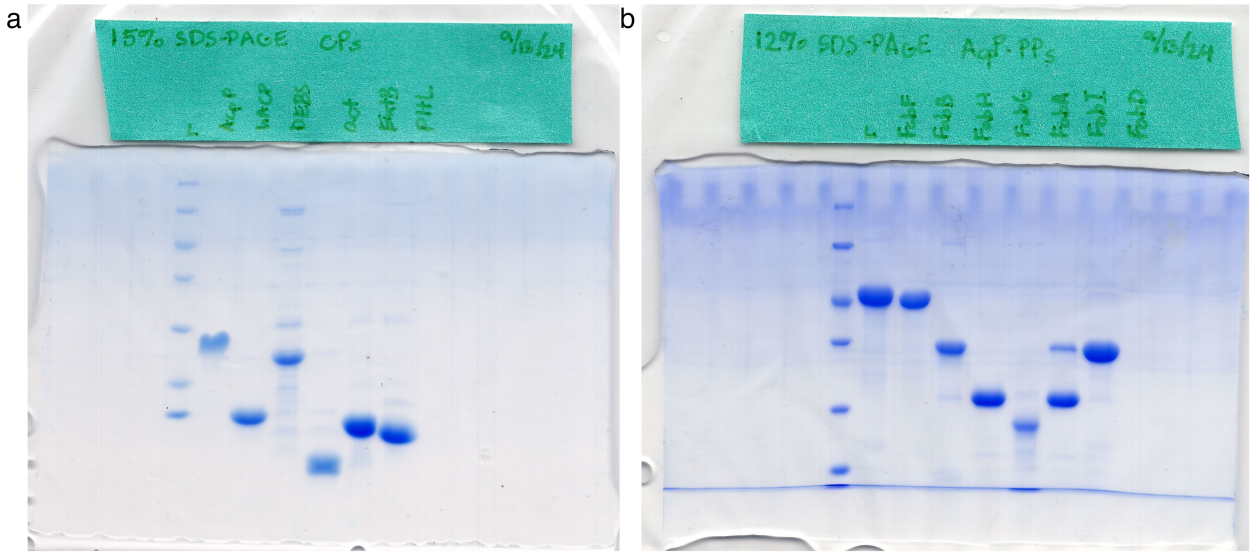


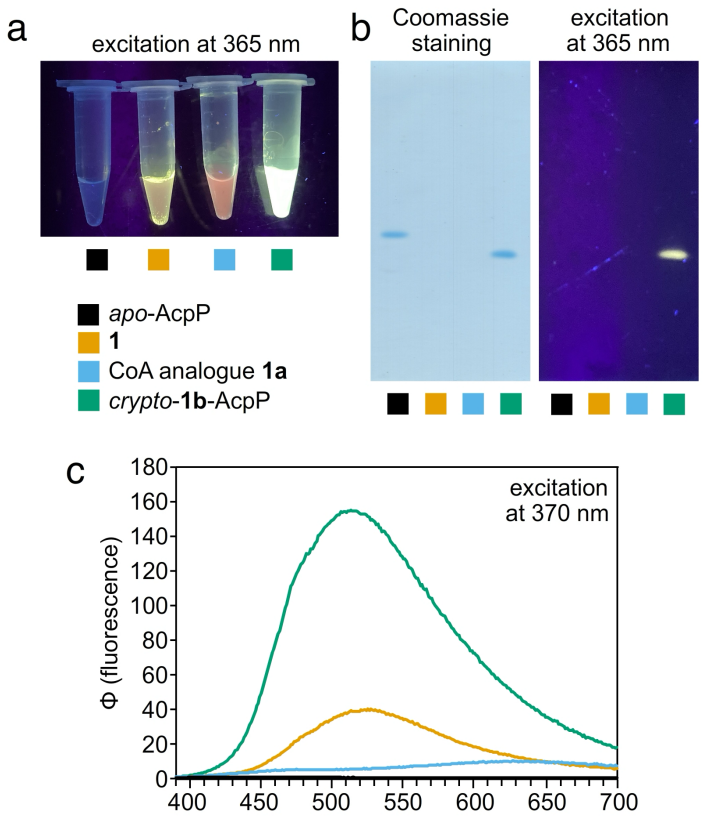
Supporting Fig. S1 SDS-PAGE analysis of purified His-tagged proteins. (a) Lanes from left to right: AcpP, hACP, DEBS Acp4, actACP, EntB, PltL. (b) Lanes from left to right: FabF, FabB, FabH, FabG, FabA, FabI, FabD. Full uncropped gels for images in this figure are provided in Supporting Fig. S3.



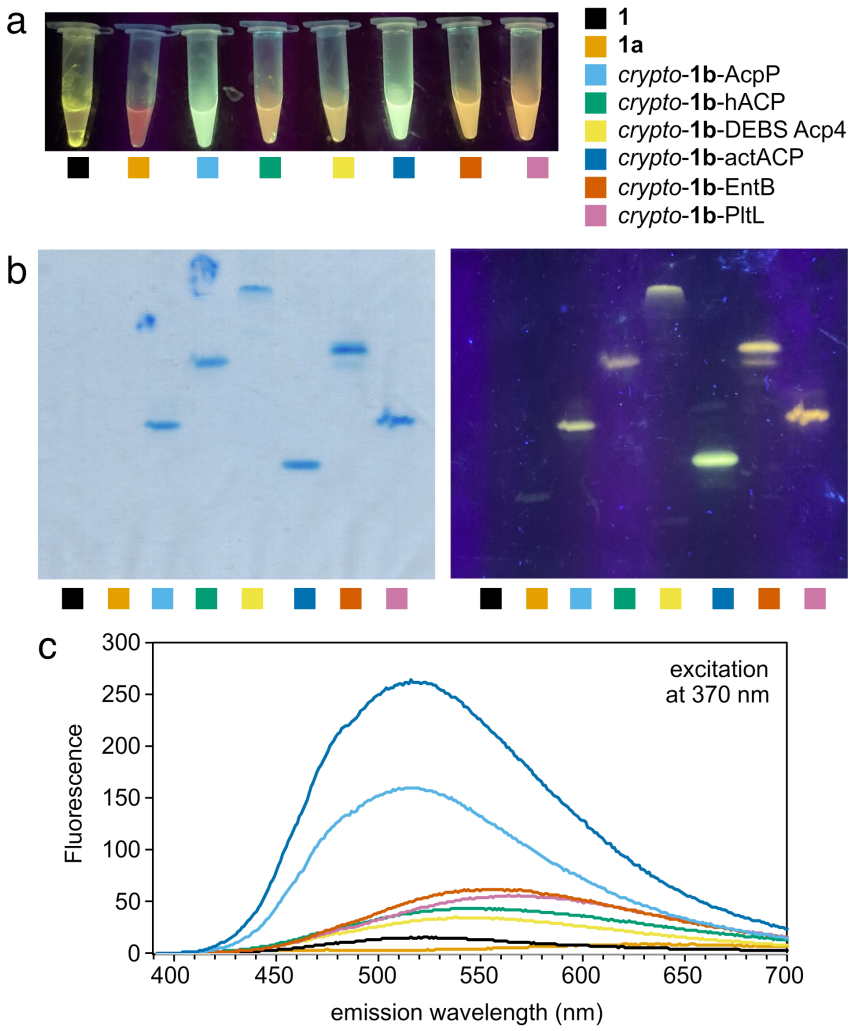
Supporting Fig. S2 Fluorometric analysis of dapoxyl-AcpP with FabF wt and R206A mutant. (a) Reaction mixtures of *crypto*-**1b**-AcpP (50 μM) loading reactions with an added 1:1 equivalent of FabF WT or R206A visualized with 365 nm UV. (b) Fluorescent emission spectra of *crypto*-**1b**-AcpP interacting with FabF WT or R206A. Excitation was at 370 nm.



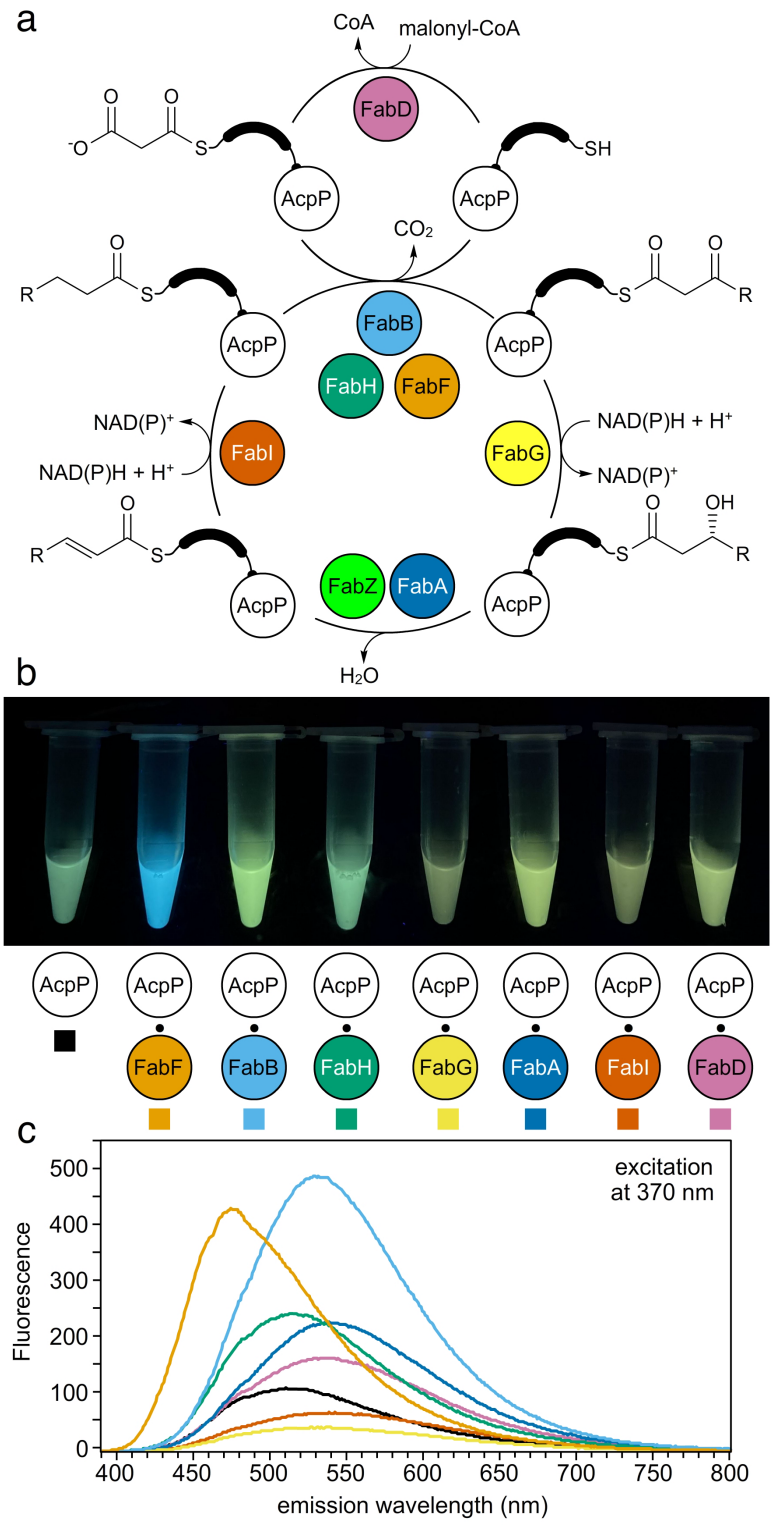
Supporting Fig. S3 Full scan of gels provided in Supporting Fig. S1.



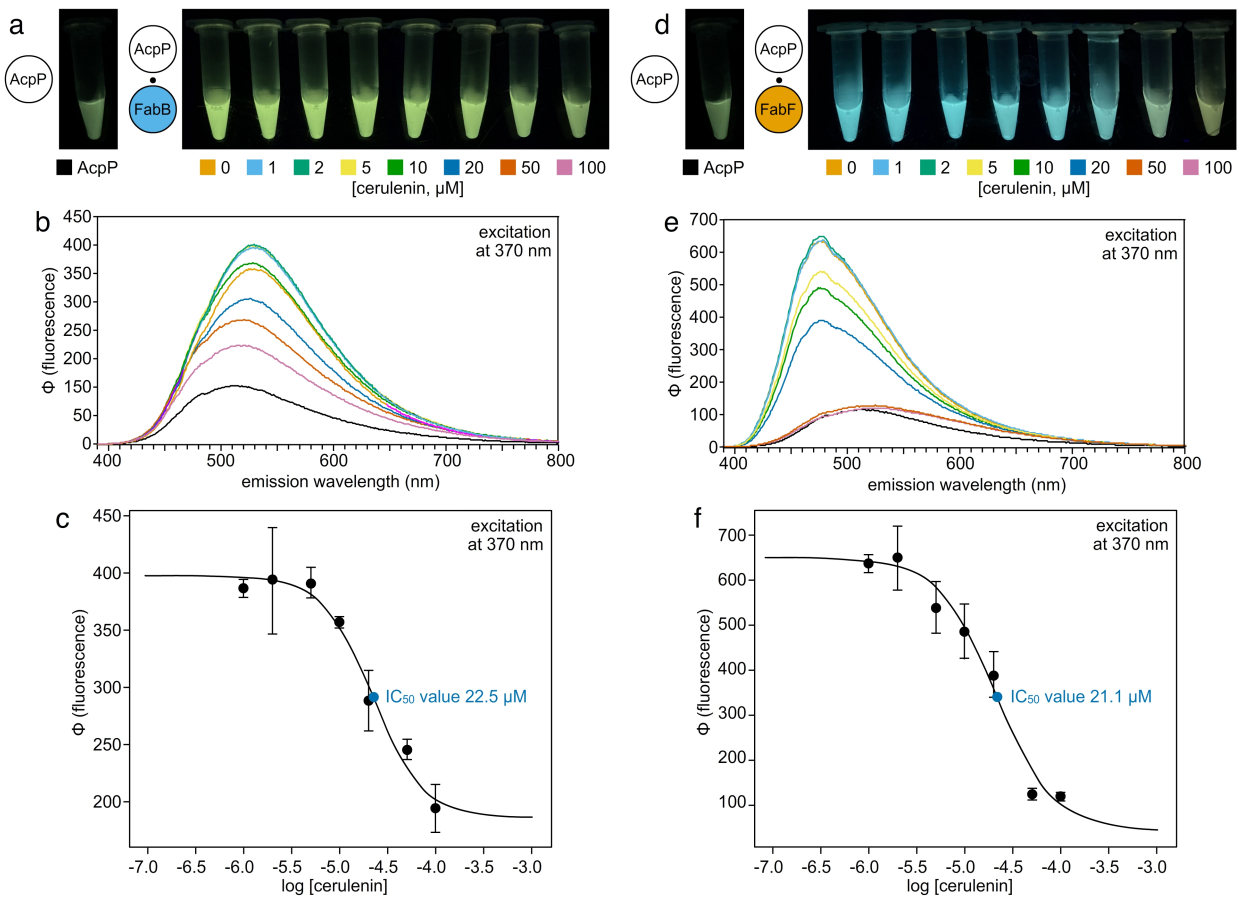
Supporting Fig. S4 Alternate color scheme used for Figure 2 according to the scheme developed in B. Wong, *Nat. Method*. 2011, **8**, 441.



Supporting Fig. S5. Alternate color scheme used for Figure 3 according to the scheme developed in B. Wong, *Nat. Method*. 2011, **8**, 441.

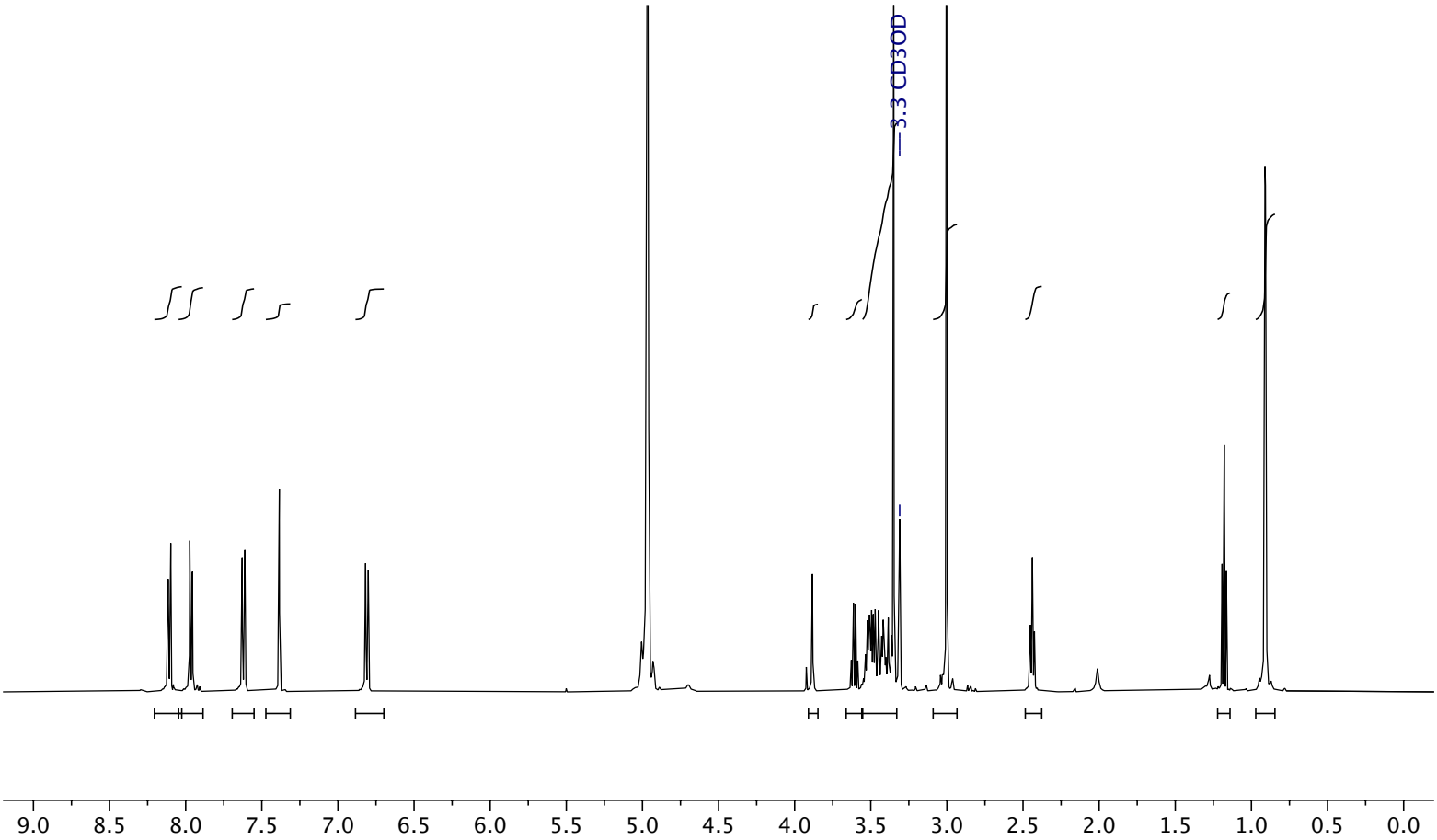


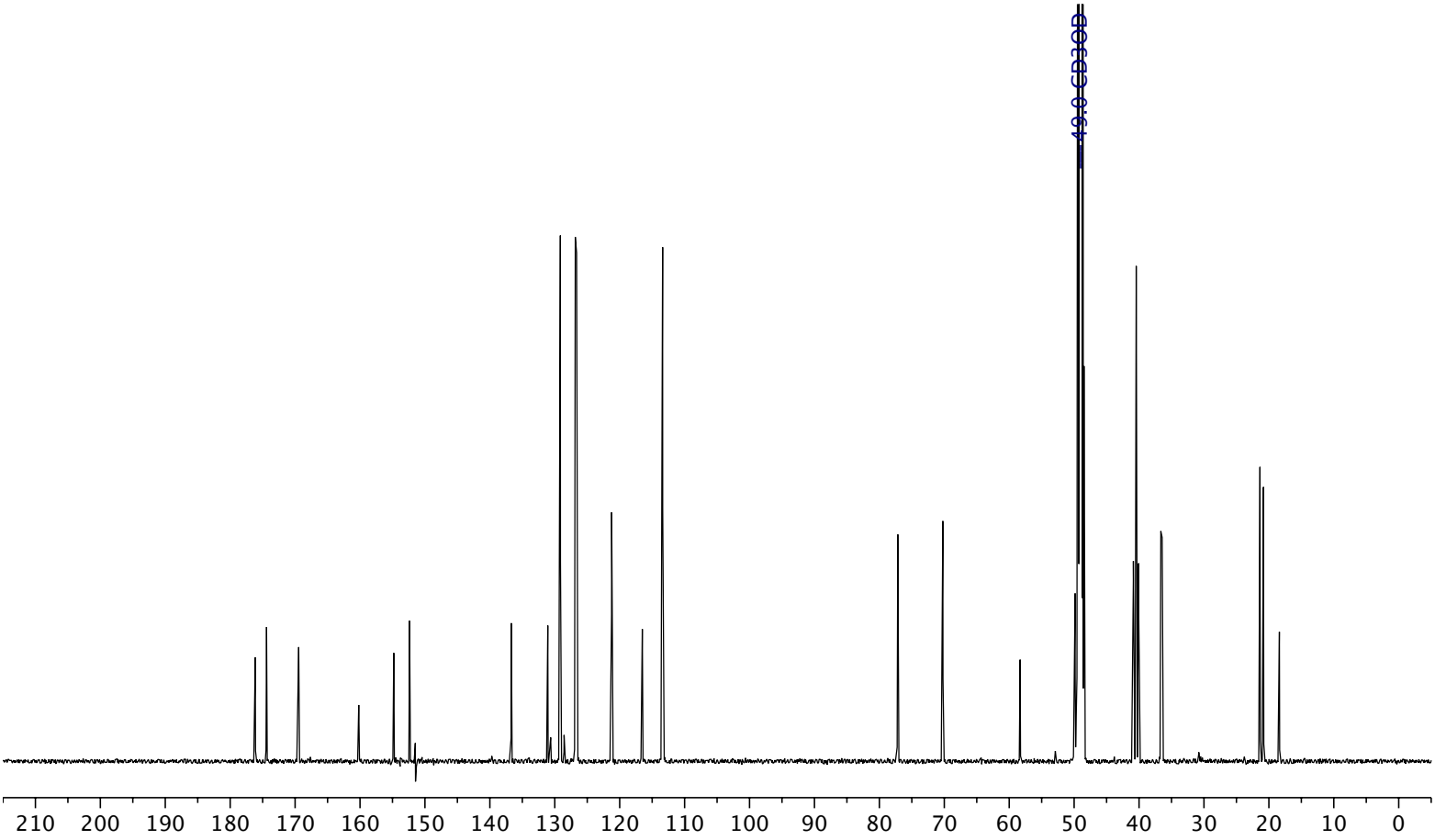
Supporting Fig. S6 Alternate color scheme used for Figure 4 according to the scheme developed in B. Wong, *Nat. Method*. 2011, **8**, 441.



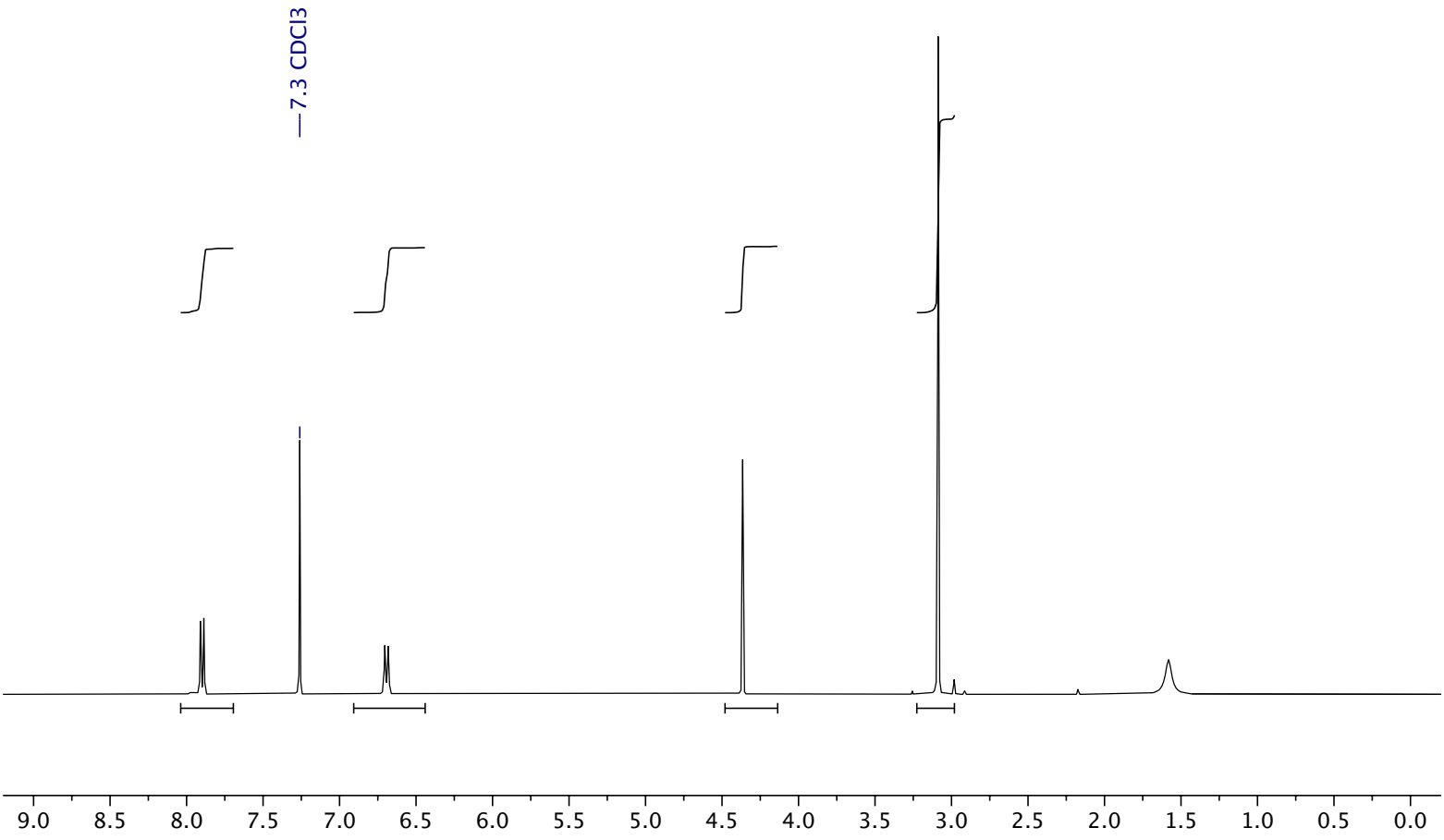
Supporting Fig. S7 Alternate color scheme used for Figure 5 according to the scheme developed in B. Wong, *Nat. Method*. 2011, **8**, 441.

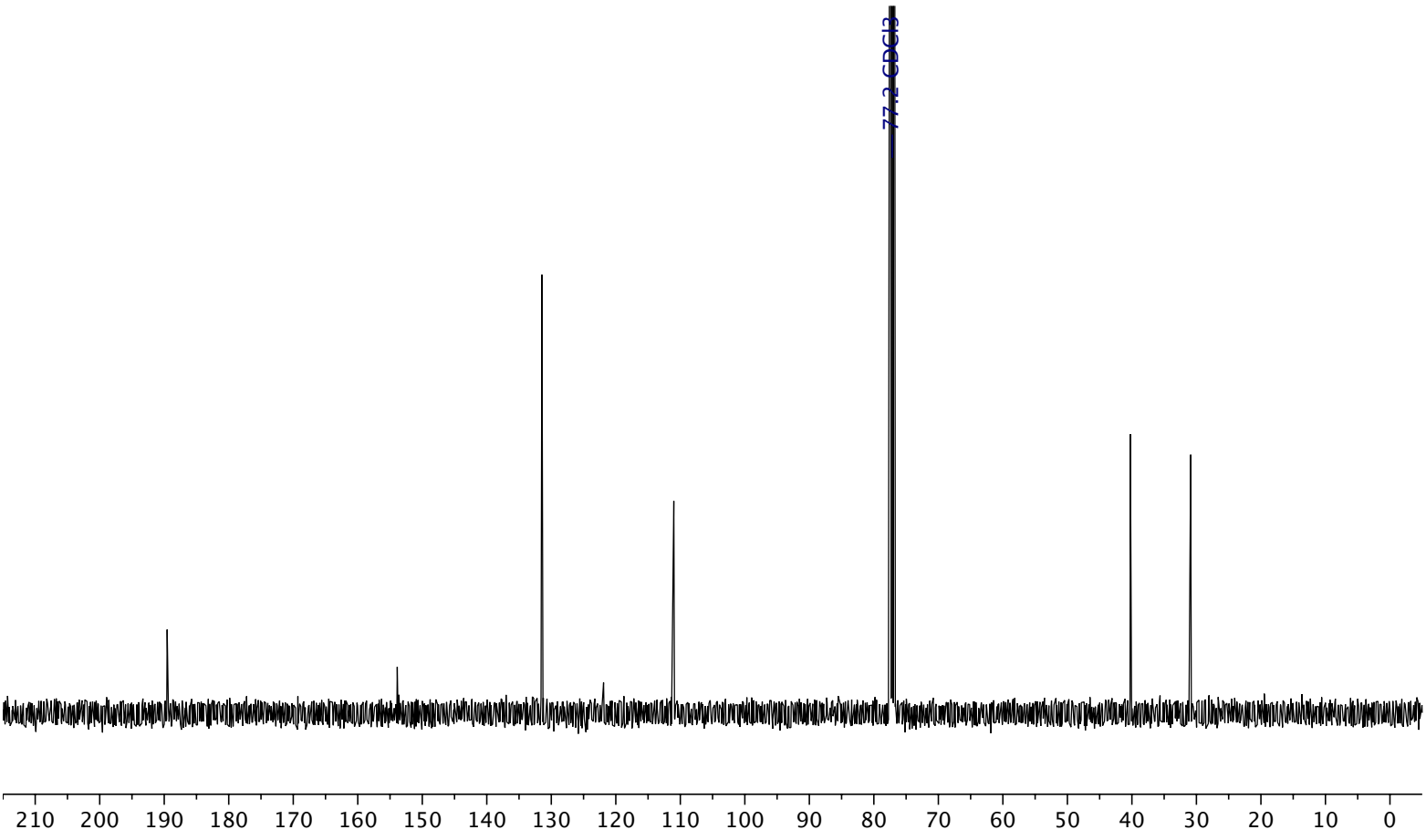
^{1}H-NMR (500 MHz) and ^{13}C-NMR (126 MHz) spectra of **1** in CD_3OD



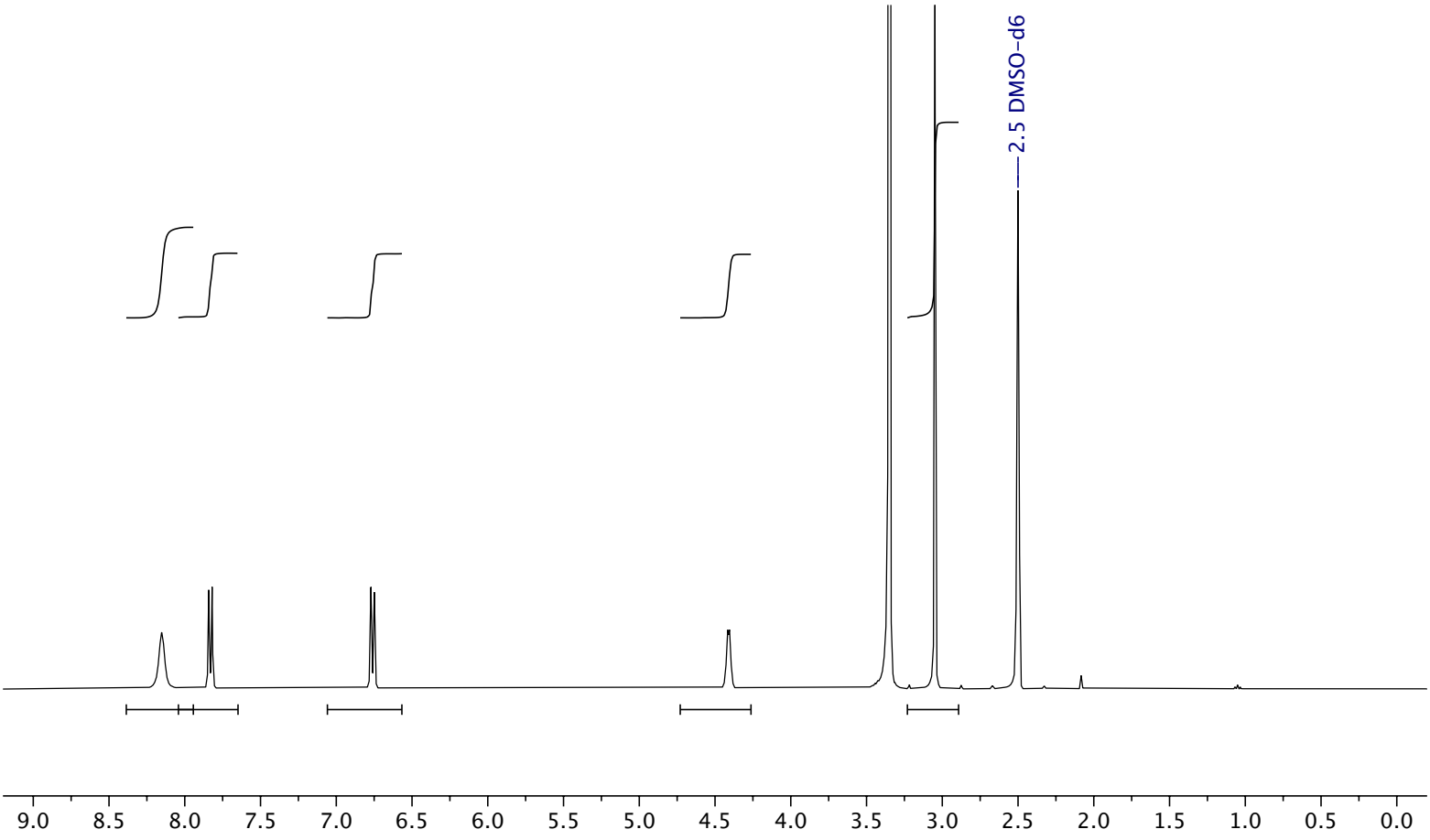


^{1}H-NMR (500 MHz) and ^{13}C-NMR (100 MHz) spectra **4** in $CDCl_3$

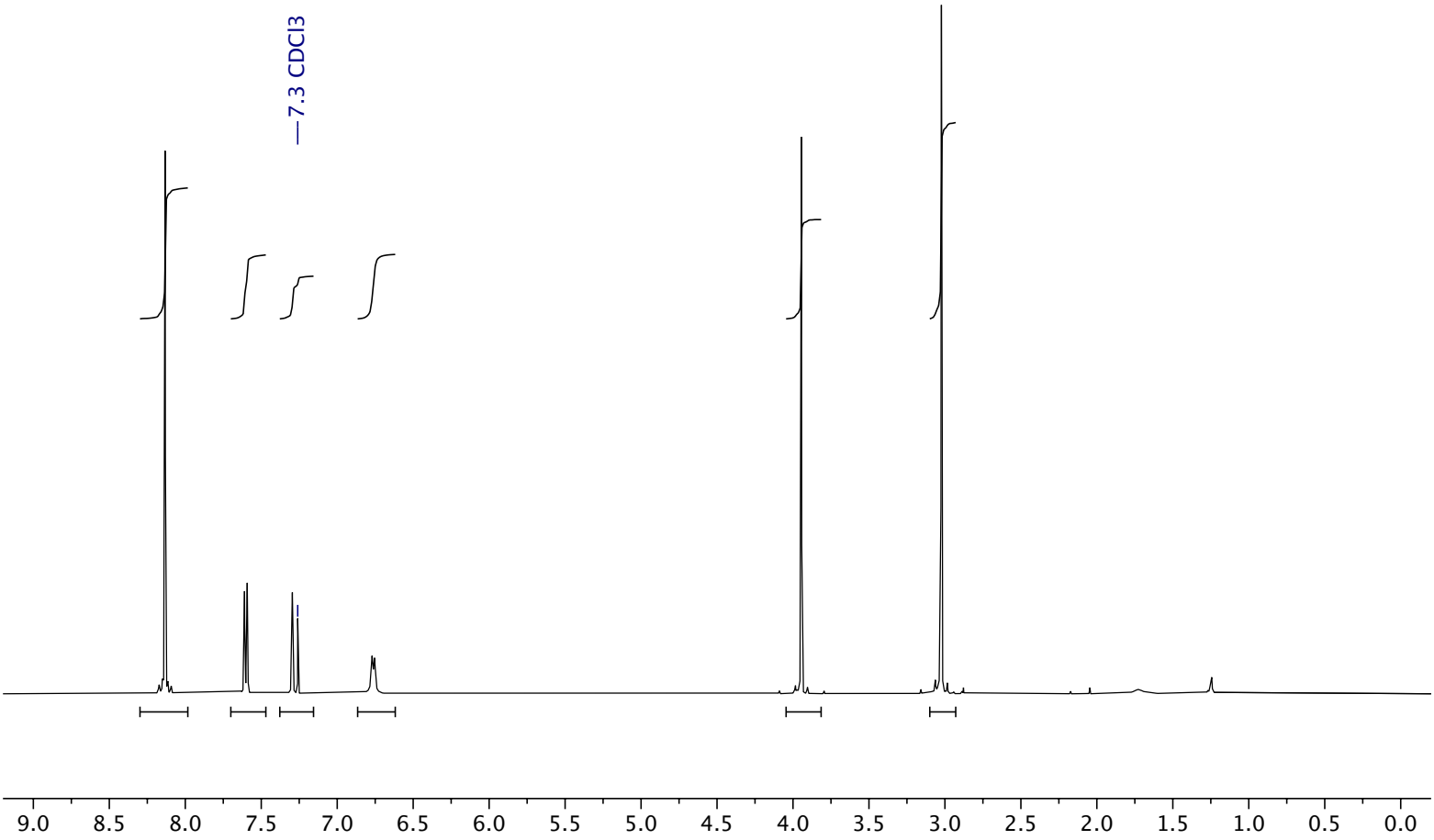


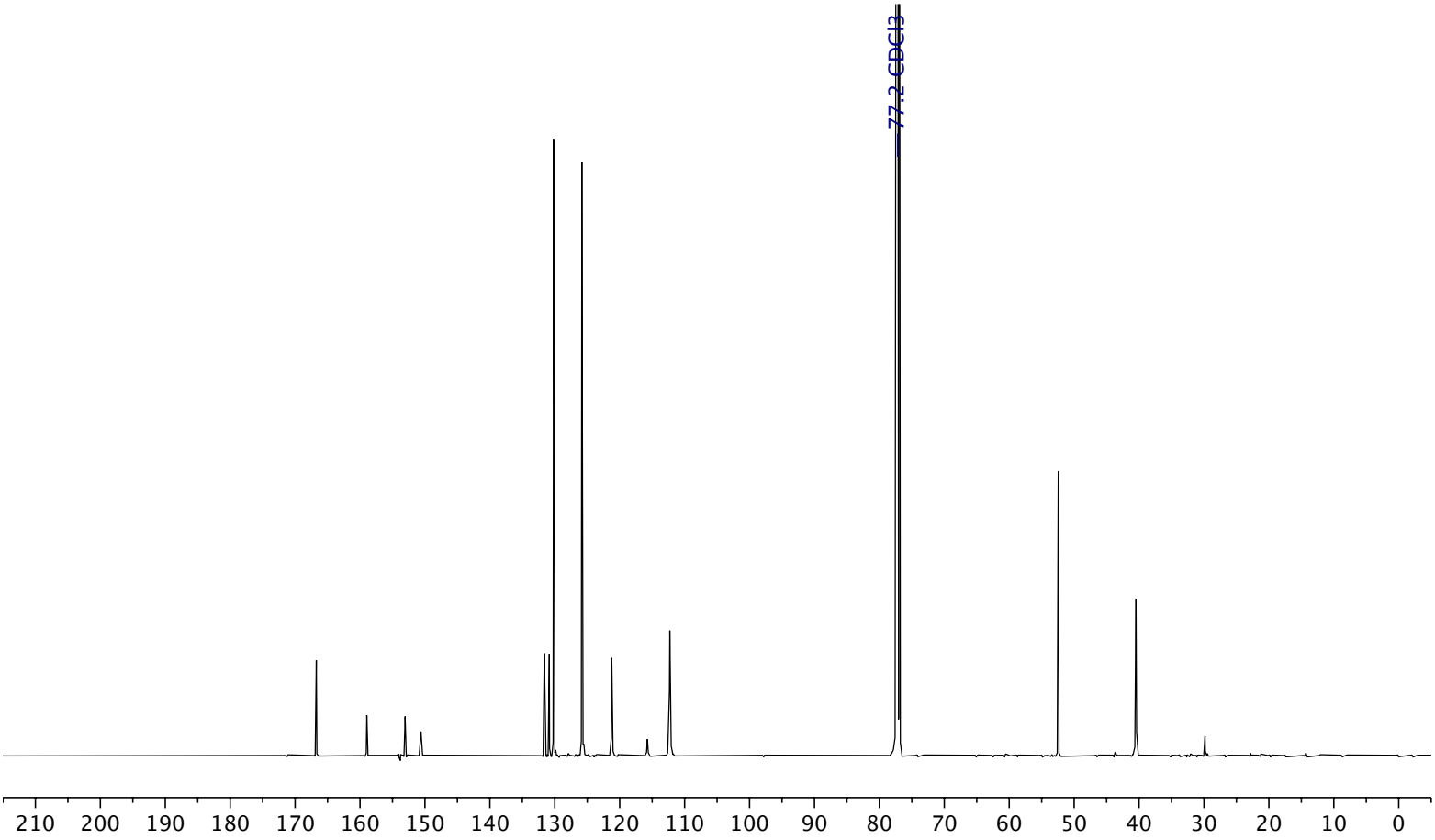


^{1}H–NMR (500 MHz) spectra of **6** in DMSO–d_6



^{1}H–NMR (500 MHz) and ^{13}C–NMR (126 MHz) spectra of **8** in $CDCl_3$





^{1}H–NMR (500 MHz) and ^{13}C–NMR (126 MHz) spectra of **11** in $CDCl_3$