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Supporting Information

Cell-Free Synthesis of Selenoproteins in High Yield and Purity for Selective Protein Tagging

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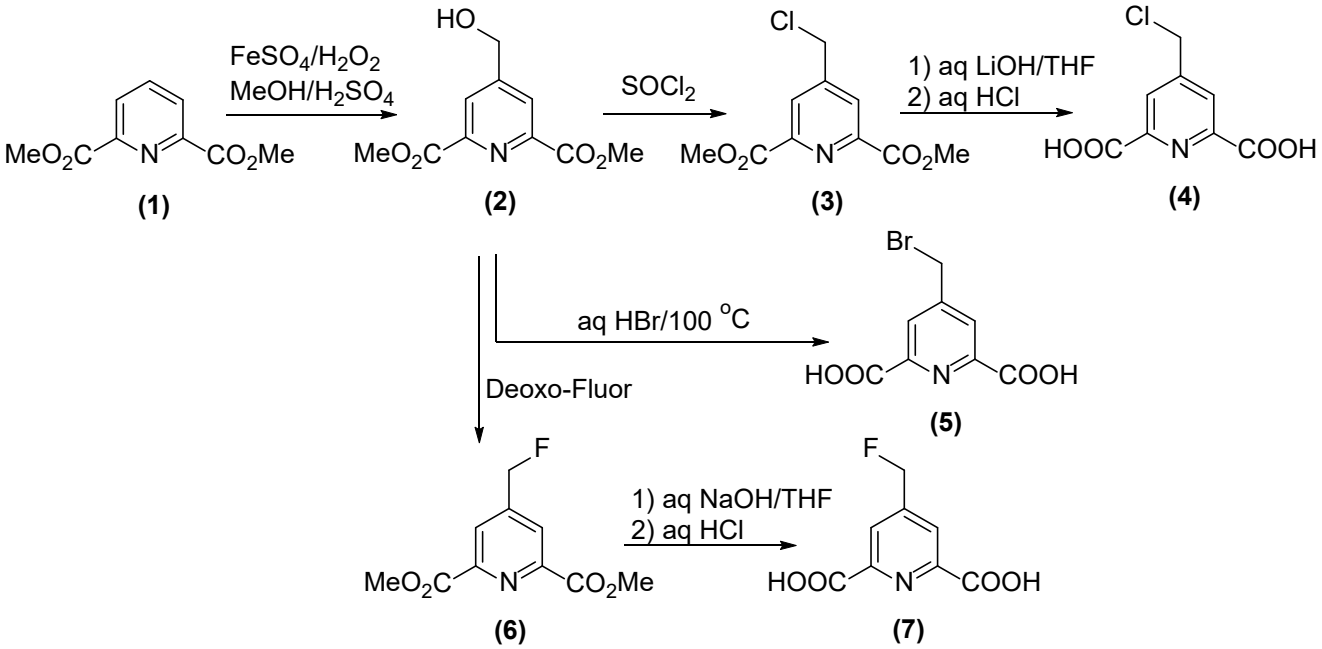

Dimethyl 4-(hydroxymethyl)pyridine-2,6-dicarboxylate (**2**) was obtained as described in the literature.[1]

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.30 (s, 2H), 4.90 (s, 2H), 4.01 (s, 6H), 2.37 (broad s, 1H) ppm.

^{13}C NMR (101 MHz, $CDCl_3$) δ: 165.28, 153.50, 148.47, 125.45, 63.04, 53.38 ppm.

HRMS (TOF ES$^+$ *m/z*): calculated for $C_{10}H_{12}NO_5$ $[M+H]^+$ 226.0715, found 226.0724.

IR (ATR): 3489 (broad s), 3080 (m), 2955 (w), 1722 (s), 1609 (m), 1451 (s), 1369 (s), 1227 (s), 1070 (m), 981 (s), 890 (m), 784 (s) cm^{-1}.

M.p.: 159 - 160 °C.

Synthesis of dimethyl 4-(chloromethyl)pyridine-2,6-dicarboxylate (**3**). Dimethyl 4-(hydroxymethyl)pyridine-2,6-dicarboxylate (**2**) (800 mg; 3.55 mmol) was dissolved in $CHCl_3$ (20 mL). $SOCl_2$ (0.73 mL; 10 mmol) was added dropwise. The reaction mixture was stirred at ambient temperature for 6 hours. TLC analysis showed complete conversion at this point. Volatiles were removed under reduced pressure and the residue was partitioned between EtOAc (50 mL) and aq. $NaHCO_3$ (100 mL). The organic phase was washed with brine, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The remaining solid was purified by silica gel column chromatography (mobile phase hexane/EtOAc with gradient from 6/1 to 1/1). 320 mg (37%) of a white solid was obtained.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.34 (s, 2H), 4.67 (s, 2H), 4.03 (s, 6H) ppm.

^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.90, 149.17, 148.99, 127.33, 53.50, 43.37 ppm.

HRMS (TOF ES$^+$ *m/z*): calculated for $C_{10}H_{11}NO_4Cl$ $[M+H]^+$ 244.0377, found 244.0388.

IR (ATR): 3072 (w), 3006 (w), 2953 (w), 1722 (s), 1606 (m), 1449 (s), 1364 (s), 1229 (s), 1154 9m), 985 (m), 890 (m) cm^{-1}.

M.p.: 151 - 153 °C.

Synthesis of 4-(chloromethyl)pyridine-2,6-dicarboxylic acid (**4**). LiOH (96 mg; 4.0 mmol) solution in water (3 mL) was added to a THF (8 mL) solution of dimethyl 4-(chloromethyl)pyridine-2,6-dicarboxylate (**3**) (320 mg; 1.3 mmol) at 0 ºC. Reaction progress was monitored by LC-MS. Upon complete conversion (~ 15 min) the reaction mixture was acidified to pH 2 with aqueous HCl (1 M). Volatiles were removed under reduced pressure. The residue was purified by reverse phase (C18) chromatography (mobile phase H_2O/MeOH with gradient 97/3 to 85/15). Fractions containing product were combined and evaporated under reduced pressure. The remaining solid was suspended in EtOAc (5 mL) and collected by filtration. After drying in vacuum, 137 mg (48%) of a slightly yellowish powder was obtained.

^{1}H NMR (400 MHz, CD_3OD) δ: 8.40 (s, 2H), 4.86 (s, 2H) ppm.

^{13}C NMR (101 MHz, CD_3OD) δ: 166.89, 152.27, 149.52, 128.20, 44.07 ppm.

HRMS (TOF ES$^+$ *m/z*): calculated for $C_8H_7NO_4Cl$ $[M+H]^+$ 216.0064, found 216.0073.

IR (ATR): 3494 (broad s), 3152 (broad s), 1728 (s), 1606 (m), 1446 (w), 1394 (w), 1338 (m), 1251 (m), 1192 (s), 1002 (w), 823 (m) cm^{-1}.

M.p.: 210 - 214 °C (with decomposition).

Synthesis of 4-(bromomethyl)pyridine-2,6-dicarboxylic acid (**5**). Dimethyl 4-(hydroxymethyl)pyridine-2,6-dicarboxylate (**2**) (500 mg; 2.22 mmol) was dissolved in aq. HBr (4 mL; 48%). The reaction mixture was heated to 100 ºC. Upon complete conversion (LC-MS control), volatiles were removed under reduced pressure. The residue was purified by reverse phase (C18) chromatography (mobile phase H_2O/MeOH with gradient from 97/3 to 85/15). Fractions containing product were combined and evaporated under reduced pressure. The remaining solid was suspended in EtOAc (5 mL) and collected by filtration. After drying in vacuum, 234 mg (40%) of a white powder was obtained.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.29 (s, 2H), 4.85 (s, 2H) ppm.

^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.41, 150.26, 149.02, 127.58, 30.59 ppm.

HRMS (TOF ES$^+$ *m/z*): calculated for $C_8H_7NO_4Br$ $[M+H]^+$ 259.9558, found 259.9562.

IR (ATR): 3461 (broad m), 3013 (broad m), 1733 (s), 1604 (w), 1441 (w), 1409 (w), 1287 (w), 1249 (m), 1175 (m) cm^{-1}.

M.p.: 216 - 218 °C (with decomposition).

Synthesis of dimethyl 4-(fluoromethyl)pyridine-2,6-dicarboxylate (**6**). Bis(2-methoxyethyl)aminosulfur trifluoride (Deoxo-Fluor® 2.7 M in toluene; 7 mL) was added to dimethyl 4-(hydroxymethyl)pyridine-2,6-dicarboxylate (**2**) (530 mg; 2.35 mmol). The reaction mixture was heated overnight to 50 ºC with intensive stirring. Next, the reaction was partitioned between EtOAc (200 mL) and sat. aq. $NaHCO_3$ (200 mL). The organic phase was washed with brine, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure.

The residue was purified by silica gel column chromatography (mobile phase hexane/EtOAc with gradient from 10/1 to 1/1). 426 mg (80%) of white solid was obtained.

^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.28 (s, 2H), 5.57 (d, *J* = 46.3 Hz, 2H), 4.04 (s, 6H) ppm.

HRMS (TOF ES$^+$ *m/z*): calculated for $C_{10}H_{11}NO_4F$ $[M+H]^+$ 228.0672, found 228.0676.

Synthesis of 4-(fluoromethyl)pyridine-2,6-dicarboxylic acid (**7**). NaOH (170 mg; 4.3 mmol) solution in water (3 mL) was added to a THF (10 mL) solution of dimethyl 4-(fluoromethyl)pyridine-2,6-dicarboxylate (**6**) (410 mg; 1.80 mmol) at 0 ºC. The reaction progress was monitored by LC-MS. Upon complete conversion (~ 15 min.), the reaction mixture was acidified to pH 2 with aqueous HCl (1 M). Volatiles were removed under reduced pressure. The residue was purified by reverse phase (C18) chromatography (mobile phase H_2O/MeOH with gradient from 97/3 to 85/15). Fractions containing product were combined and evaporated under reduced pressure. The remaining solid was suspended in EtOAc (5 mL) and collected by filtration. After drying in vacuum 167 mg (46%) of a white powder was obtained.

^{1}H NMR (300 MHz, CD_3CN) δ: 8.31 (apparent q, *J* = 0.7 Hz, 2H), 5.64 (dt, *J* = 46.3, 0.7 Hz, 2H) ppm.

^{19}F NMR (376 MHz, CD_3CN) δ: -223.02 (t, *J* = 46.3 Hz) ppm.

^{13}C NMR (101 MHz, CD_3CN) δ: 164.61, 151.24 (d, *J* = 18.9 Hz), 147.49, 125.09 (d, *J* = 7.8 Hz), 82.51 (d, *J* = 168.7 Hz) ppm.

HRMS (TOF ES$^+$ *m/z*): calculated for $C_8H_7NO_4F$ $[M+H]^+$ 200.0359, found 200.0361.

IR (ATR): 3501 (broad s), 3090 (broad s), 1921 (broad m), 1728 (s), 1610 (m), 1367 (m), 1333 (m), 1265 (m), 1252 (m), 1191 (s), 1131 (m), 1039 (m), 848 (m) cm^{-1}.

M.p.: 212 - 214 °C (with decomposition).

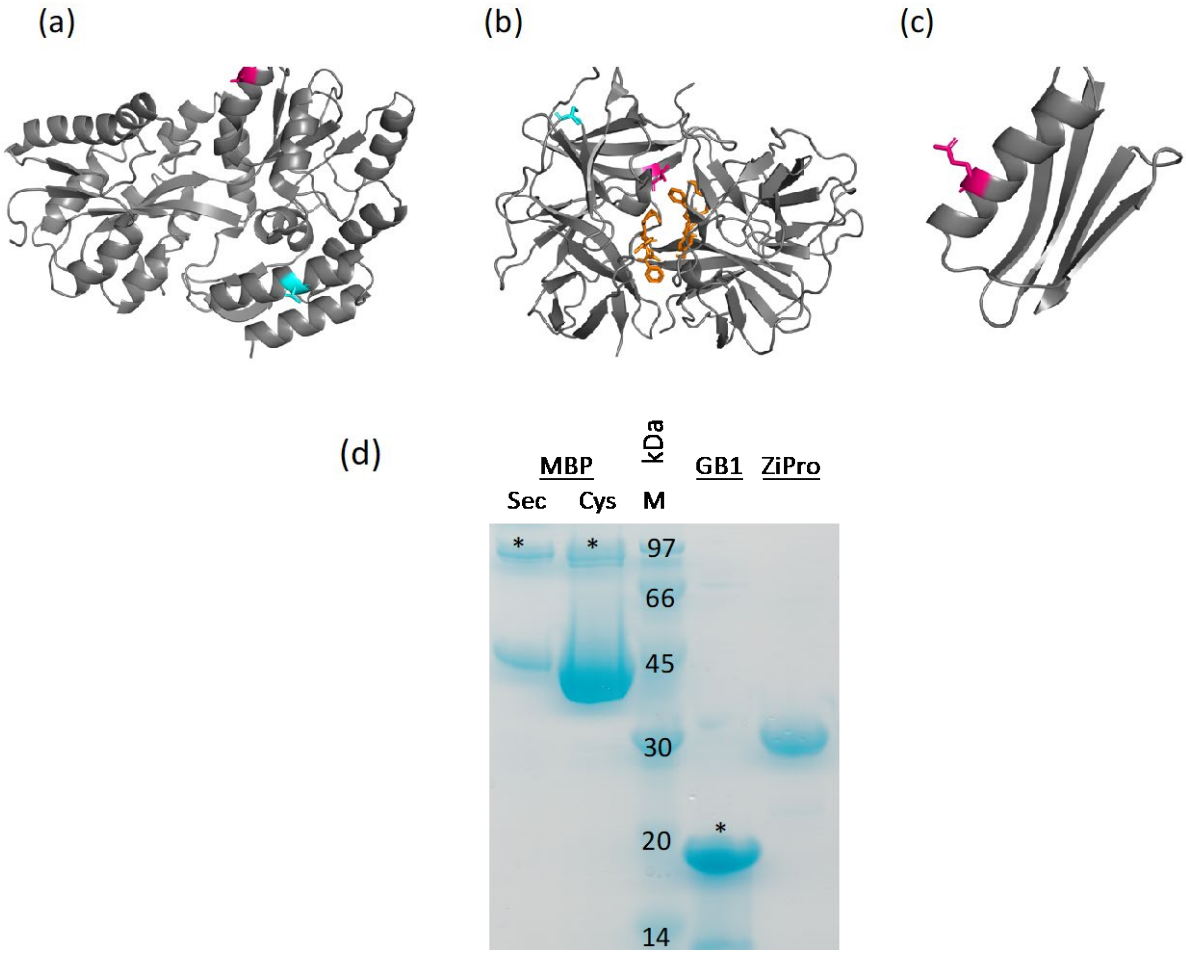

Figure S1: Crystal structures of the protein mutants. (a) Crystal structure of MBP (PDB ID: 1OMP). T237 and T345 are shown in pink and cyan. (b) Crystal structure of ZiPro (PDB ID: 5LC0) with bound inhibitor (orange). V36 and C80 are shown in pink and cyan. (c) Crystal structure of GB1 (PDB ID: 1PGA). Q32 is shown in pink. (d) SDS-PAGE analysis of purified MBP T237U/T345U, MBP T237C/T345C, GB1 Q32U, ZiPro V36U after purification on a Co-NTA column. Dimers of purified MBP T237U/T345U, MBP T237C/T345C and GB1 Q32U are shown with star marks. M: Marker.

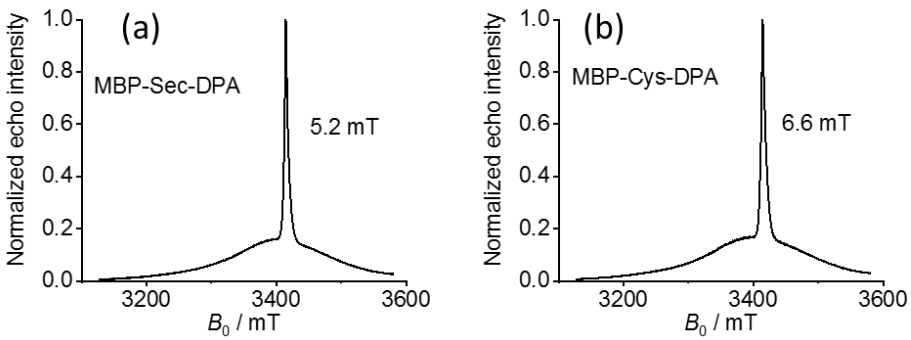

Figure S2. W-band ED-EPR spectra for the Sec (a) and Cys (b) MBP mutants. The full width at half-height (FWHH) of the central transition is given.

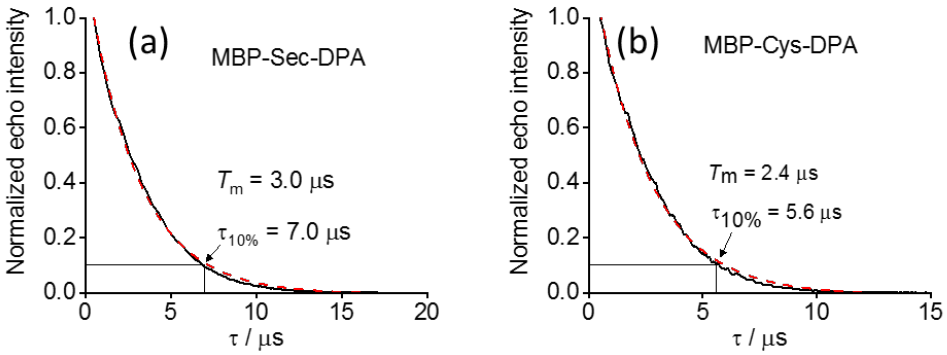

Figure S3. Echo decay traces for the Sec (a) and Cys (b) MBP mutants. The phase memory time T_m after fitting a mono-exponential decay and the time at which the echo intensity was reduced to 10% of its initial value ($\tau_{10\%}$) are given.

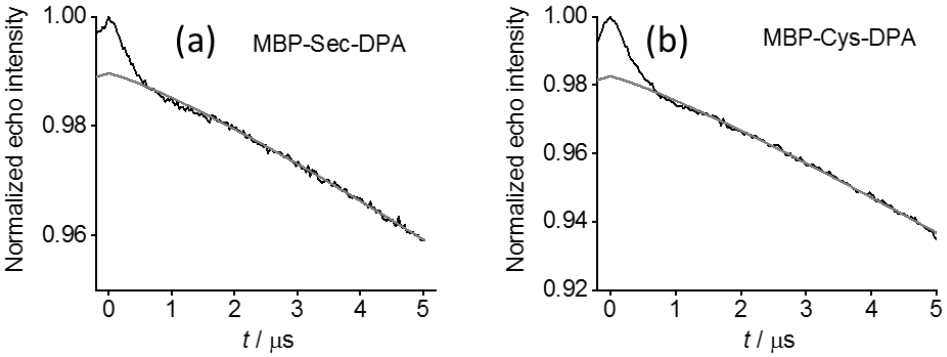

Figure S4. Primary Gd(III)-Gd(III) DEER data for the Sec (a) and Cys (b) MBP mutants with the removal of the background indicated by a gray line.

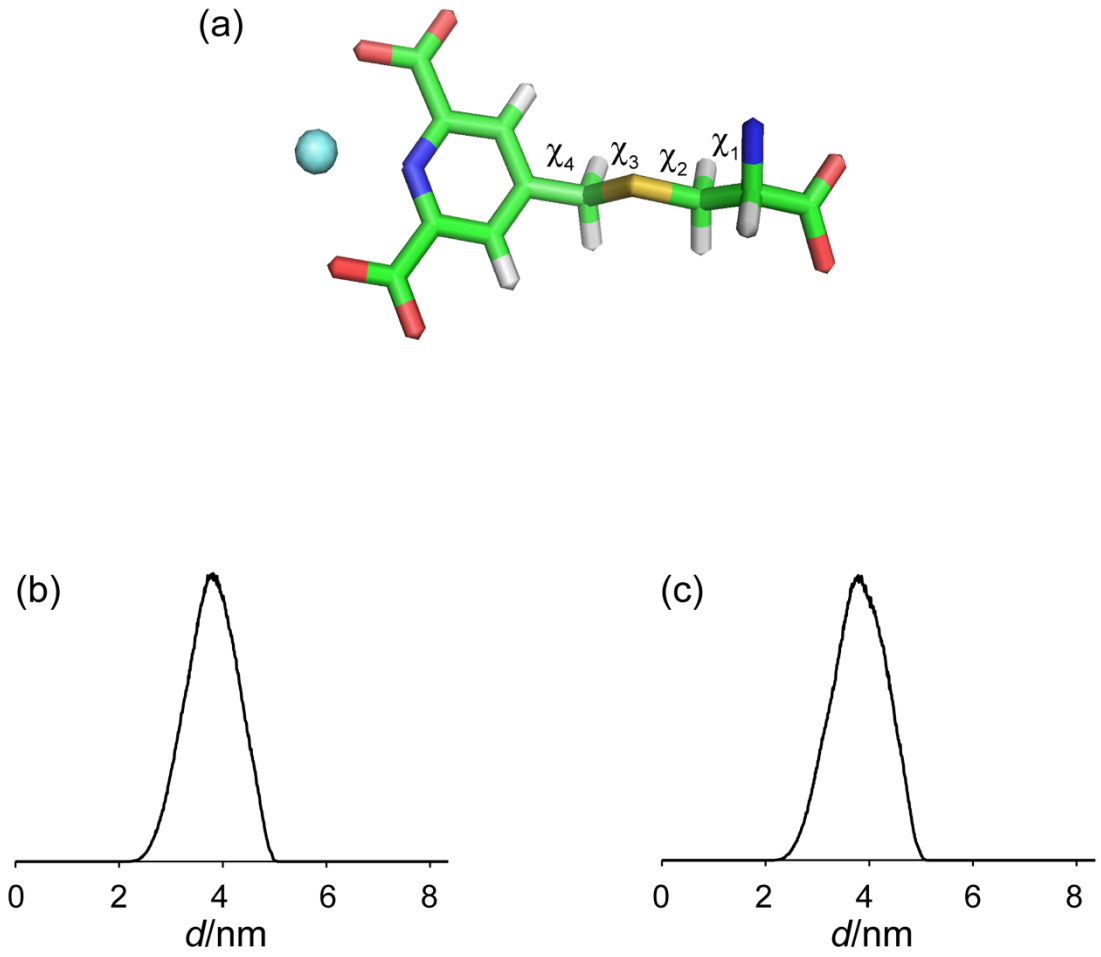

Figure S4. Modelling of DEER distance distributions. The program PyParaTools[2] was used to generate many different tag conformations on the crystal structure 1OMP of maltose binding protein[3]. (a) Chemical structure of the cysteine residue with MDPA tag attached. The lanthanide ion is depicted as a cyan sphere. The dihedral angles identified in the figure were varied in random combinations to generate rotamer libraries, allowing the dihedral angles to vary completely randomly (χ_2, χ_3, and χ_4) or within ranges of ±10° of staggered conformations 60°, -60°, and 180° (χ_1). (b) Distance distributions modelled for MBP with MDPA tags attached to Cys237 and Cys345. (c) Same as (b), except with MDPA tags attached to selenocysteine in positions 237 and 345.

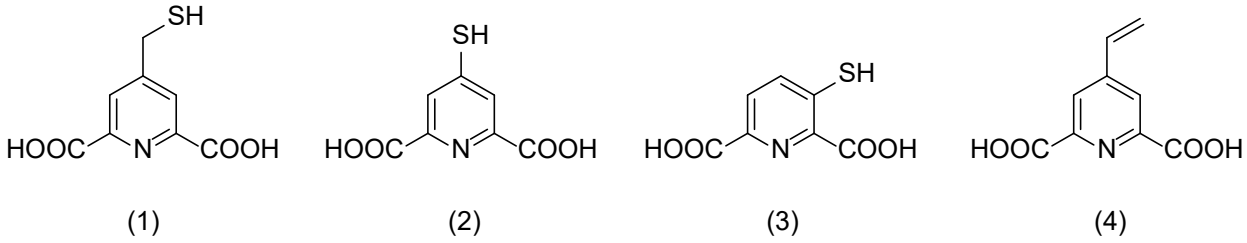

Figure S5. Structures of lanthanide binding tags mentioned in the main text but not used in this study: (1) 4-mercaptomethyl-dipicolinic acid (4MMDPA); (2) 4-mercaptodipicolinic acid (4MDPA); (3) 3-mercaptodipicolinic acid (3MDPA); (4) 4-vinyl-DPA.

References

(1) Schmidt, A.-C., Hermsen, M., Rominger, F., Dehn, R., Teles, J. H., Schafer, A., Trapp, O., Schaub, T. (2017) Synthesis of mono- and dinuclear vanadium complexes and their reactivity toward dehydroperoxidation of alkyl hydroperoxides. *Inorg. Chem. 56*, 1319–1332.

(2) M. Stanton-Cook, X.-C. Su, G. Otting and T. Huber, PyParaTools – Software for working with paramagnetic NMR data, http://comp-bio.anu.edu.au/mscook/PPT/, (accessed September 21, 2020).

(3) A. J. Sharff, L. E. Rodseth, J. C. Spurlino and F. A. Quiocho (1992) Crystallographic evidence of a large ligand-induced hinge-twist motion between the two domains of the maltodextrin binding protein involved in active transport and chemotaxis. *Biochemistry 31*, 10657–10663.