# Supporting Information

## The Genetic Incorporation of a Small, Environmentally Sensitive, Fluorescent Probe into Proteins in *S. Cerevisiae*

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## Materials and Methods

**General**. All chemicals and DNA oligomers were obtained from commercial sources and used without further purification. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker DRX-500 instrument with chemical shifts recorded relative to tetramethylsilane. Protein mass spectra were acquired at the Scripps Center for Mass Spectrometry (La Jolla, CA).

**Synthesis of unnatural amino acids.**

**Ethyl 3-((6-acetylnaphthalen-2-yl)(2,4-dimethoxybenzyl)amino)-2-(hydroxyimino) propanoate.** To a stirred suspension of 1-(6-hydroxynaphthalen-2-yl)ethanone (*S1*) (4.0 g, 21 mmol) and sodium metabisulfite (12.4 g, 65 mmol) in water (95 mL) was added 2,4-dimethoxybenzylamine (7.3 g, 43 mmol) and the mixture was stirred for 24 h at 140 °C in a 150 mL pressure vessel. The reaction mixture was cooled to room temperature and the aqueous phase discarded. The oily residue was dissolved in methylene chloride and washed once with saturated sodium bicarbonate solution. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The residue was dissolved in DMF (60 mL), and ethyl 3-bromo-2-(hydroxyimino)propanoate (*S2*) (4.4 g, 21 mmol) and cesium carbonate (7.5 g, 23 mmol) were added. The mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with ethyl acetate and washed twice with 10% aqueous sodium thiosulfate solution. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by column

chromatography (silica, 30% ethyl acetate in hexane) to give the product (2.5 g, 26%) as a yellow solid. $^{1}H$-NMR (500 MHz, $CD_3OD$) : δ 1.18 (t, 3H), 2.66 (s, 3H), 3.76 (s, 3H), 3.91 (s, 3H), 4.17 (q, 2H), 4.69 (s, 2H), 4.72 (s, 2H), 6.43 (dd, 1H), 6.66 (d, 1H), 6.89 (d, 1H), 7.01 (d, 1H), 7.22 (dd, 1H), 7.59 (d, 1H), 7.85 (dd, 1H), 7.93 (d, 1H), 8.47 (d, 1H). $^{13}C$-NMR (500 MHz, $CD_3OD$) : δ 14.7, 27.3, 45.6, 50.7, 56.0, 56.3, 61.9, 99.4, 105.2, 106.0, 117.2, 118.1, 124.9, 125.6, 126.7, 128.3, 131.1, 131.2, 131.5, 137.9, 148.6, 150.0, 158.7, 160.5, 164.3, 197.8. LC-MS (ESI) calcd for $C_{26}H_{28}N_2O_6$ (M+1) 465.2, obsd. 465.2.

**Ethyl 3-((2,4-dimethoxybenzyl)(6-(1-hydroxyethyl)naphthalen-2-yl)amino)-2-(hydroxyl-mino)propanoate.** To a stirred suspension of compound **4** (2.5 g, 5.4 mmol) in EtOH was added sodium borohydride (1.5 g, 40 mmol) and the mixture was stirred for 2 h at room temperature. The suspension became clear as the reaction proceeded. The reaction mixture was concentrated under reduced pressure, dissolved in ethyl acetate and washed once with saturated aqueous sodium bicarbonate solution. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure to give the product (2.4 g, 96%) as a foamy solid. $^{1}H$-NMR (500 MHz, $CDCl_3$) : δ 1.29 (t, 3H), 1.62 (d, 3H), 3.85 (s, 3H), 3.93 (s, 3H), 4.26 (q, 2H), 4.68 (s, 2H), 4.76 (s, 2H), 5.06 (q, 1H), 5.37 (s, 1H), 6.37 (dd, 1H), 6.56 (d, 1H), 6.97 (d, 1H), 7.11 (d, 1H), 7.23 (dd, 1H), 7.40 (d, 1H), 7.62 (d, 1H), 7.68 (m, 2H). $^{13}C$-NMR (500 MHz, $CDCl_3$) : δ 14.3, 25.2, 46.0, 51.5, 55.7, 55.8, 62.3, 71.1, 98.9, 99.0, 100.0, 104.1, 117.2,118.5, 123.9, 124.5, 127.3, 127.4, 128.8, 129.3, 134.8, 139.9, 146.4, 158.7, 160.4, 163.7. LC-MS (ESI) calcd for $C_{26}H_{30}N_2O_6$ (M+1) 467.2, obsd. 467.3.

**Ethyl 2-amino-3-((2,4-dimethoxybenzyl)(6-(1-hydroxyethyl)naphthalen-2-yl)amino) pro-panoate.** To a stirred solution of ethyl 3-((2,4-dimethoxybenzyl)(6-(1-hydroxyethyl) naphthalen-2-yl)amino)-2-(hydroxyimino)propanoate (2.4 g, 5.1 mmol) in THF/water (80 mL/8 mL) was added aluminum amalgam prepared from aluminum foil (2.0 g) and mercuric chloride (100 mg) in water (100 mL) at room temperature (*S3*). The reaction was monitored by TLC until the starting material had been consumed (about 1 h). The mixture was then filtered through Celite, the filter pad washed well with ethyl acetate, and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography (silica, 50% ethyl acetate in methylene chloride to 100% ethyl acetate) to

give the product (2.0 g, 86%) as a foamy solid. $^{1}$H-NMR (500 MHz, $CDCl_3$) : δ 1.33 (t, 3H), 1.63 (d, 3H), 3.77 (d, 2H), 3.83 (s, 3H), 3.93 (s, 3H), 4.03 (t, 1H), 4.23 (q, 2H), 4.73 (s, 2H), 5.07 (q, 1H), 5.38 (s, 1H), 6.39 (dd, 1H), 6.57 (d, 1H), 6.97 (d, 1H), 7.07 (d, 1H), 7.23 (dd, 1H), 7.45 (dd, 1H), 7.67 (d, 1H), 7.72 (m, 2H). $^{13}$C-NMR (500 MHz, $CDCl_3$) : δ 14.6, 25.3, 30.2, 53.9, 55.7, 55.8, 56.4, 63.2, 71.0, 98.9, 100.0, 104.1, 106.9, 116.8, 118.2, 1 23.9, 124.6, 126.9, 127.1, 128.4, 129.3, 134.9, 139.8, 146.8, 160.3, 175.1. LC-MS (ESI) calcd for $C_{26}H_{32}N_2O_5$ (M+1) 453.2, obsd. 453.3.

**Ethyl 2-(*tert*-butoxycarbonylamino)-3-((2,4-dimethoxybenzyl)(6-(1-hydroxyethyl)naphthalen-2-yl)amino)propanoate.** To a stirred solution of compound **5** (1.8 g, 4.0 mmol) and triethylamine (1.1 mL, 8.0 mmol) in methylene chloride (40 mL) was added di-*tert*-butyl dicarbonate (1.3 g, 6.0 mmol) and the mixture was stirred for 12 h at room temperature. The reaction mixture was directly purified by column chromatography (silica, 50% ethyl acetate in hexane) to give the product (1.6 g, 73%) as a foamy solid. $^{1}$H-NMR (500 MHz, $CDCl_3$) : δ 1.27 (t, 3H), 1.48 (s, 9H), 1.63 (d, 3H), 3.83 (s, 3H), 3.92 (s, 3H), 4.00-4.20 (m, 2H), 4.21 (q, 2H), 4.64 (dd, 2H), 4.74 (br, 1H), 5.06 (q, 1H), 5.33 (d, 1H), 6.39 (dd, 1H), 6.57 (d, 1H), 6.96 (d, 1H), 7.07 (s, 1H), 7.22 (dd, 1H), 7.45 (d, 1H), 7.65 (d, 1H), 7.71 (m, 2H). $^{13}$C-NMR (500 MHz, $CDCl_3$) : δ 14.4, 14.6, 21.5, 25.4, 28.7, 50.3, 53.2, 53.4, 55.6, 55.7, 60.8, 62.1, 71.0, 98.9, 104.1, 107.2, 116.7, 118.1, 123.9, 124.6, 127.1, 128.5, 129.3, 134.9, 139.8, 1 46.7, 155.5, 158.6, 160.3, 172.2. LC-MS (ESI) calcd for $C_{31}H_{40}N_2O_7$ (M+1) 553.3, obsd. 553.3.

**Ethyl 3-((6-acetylnaphthalen-2-yl)(2,4-dimethoxybenzyl)amino)-2-(tert-butoxycarbonylamino)propanoate.** To a stirred solution of ethyl 2-(*tert*-butoxycarbonylamino)-3-((2,4-dimethoxybenzyl)(6-(1-hydroxyethyl)naphthalen-2-yl)amino)propanoate (1.5 g, 2.7 mmol) in acetone (50 mL) was added manganese (IV) oxide (7.5 g, activated, ~85%, <5μm) and the mixture was stirred for 12 h at room temperature. The reaction mixture was filtered on celite and concentrated under reduced pressure. The residue was purified by column chromatography (silica, 30% ethyl acetate and 10% $CHCl_3$ in hexane) to give the product (1.2 g, 80%) as a yellow solid. $^{1}$H-NMR (500 MHz, $CDCl_3$) : δ 1.29 (t, 3H), 1.47 (s, 9H), 2.74 (s, 3H), 3.86 (s, 3H), 3.95 (s, 3H), 3.98-4.15 (m, 2H), 4.21 (q, 2H), 4.70 (dd, 2H), 4.77 (br, 1H),

5.34 (br, 1H), 6.41 (dd, 1H), 6.58 (d, 1H), 6.95 (d, 1H), 7.08 (s, 1H), 7.26 (dd, 1H), 7.65 (d, 1H), 7.83 (d, 1H), 7.97 (dd, 1H), 8.36 (s, 1H). $^{13}$C-NMR (500 MHz, $CDCl_3$) : δ 14.4, 26.8, 28.7, 50.2, 52.9, 53.3, 55.6, 55.7, 55.8, 58.4, 62.3, 80.5, 99.0, 100.0, 104.1,106.4, 116.7, 117.4, 124.9, 125.8, 126.8, 128.2, 130.5, 130.6, 131.3, 138.0, 155.5, 158.6, 160.5, 198.1. LC-MS (ESI) calcd for $C_{31}H_{38}N_2O_7$ (M+1) 551.3, obsd. 551.3.

**Ethyl 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoate (1).** To a stirred solution of compound **7** (1.2 g, 2.2 mmol) in $CH_2Cl_2$ (15 mL) was added trifluoacetic acid (15 mL) and the mixture was stirred for 2 h at room temperature. The reaction mixture was concentrated under reduced pressure, diluted with ethyl acetate and washed once with saturated aqueous sodium bicarbonate solution. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The residue was dissolved in ethanol (20 mL) and 5 N aqueous sodium hydroxide solution (0.48 mL) was added. The mixture was stirred for 2 h at room temperature. The yellow precipitate was collected by filtering and washed with 50% ethanol in diethyl ether to give a yellow solid (0.42 g, 65%) in racemic form as a sodium salt. $^{13}$H-NMR (500 MHz, DMSO-$d_6$) : δ 1.85 (br, 2H), 2.66 (s, 3H), 3.12 (m, 1H), 3.24 (m, 1H), 3.30 (m, 1H), 6.53 (t, 1H), 6.79 (d, 1H), 7.11 (dd, 1H), 7.64 (d, 1H), 7.83 (dd, 2H), 8.41 (d, 1H). $^{13}$C-NMR (500 MHz, DMSO-$d_6$) : δ 27.2, 49.9, 55.5, 100.0, 102.7, 120.1, 124.9, 125.6, 126.2, 130.3, 131.3, 138.9, 150.3, 177.3, 197.7. LC-MS (ESI) calcd for $C_{15}H_{16}N_2O_3$ (M+1) 273.1, obsd. 273.1.

**3-(Naphthalen-2-ylamino)-2-aminopropanoic acid (2).** To a stirred solution of 2-aminonaphthalene (2.9 g, 20 mmol) in DMF (60 mL) was added ethyl 3-bromo-2-(hydroxyimino)propanoate (*S2*) (4.6 g, 22 mmol) and cesium carbonate (7.2 g, 22 mmol). The mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with ethyl acetate and washed twice with 10% aqueous sodium thiosulfate solution. The crude product was used for the next step without purification. To a stirred solution of the product in THF/water (150 mL/15 mL) was added aluminum amalgam prepared from aluminum foil (8.0 g) and mercuric chloride (200 mg) in water (200 mL) at room temperature (*S3*). The reaction was monitored by TLC until the starting material had been consumed (about 1 h). The mixture was then filtered through Celite, the filter pad washed well with ethyl acetate, and the

filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography (silica, 50% ethyl acetate in methylene chloride to 100% ethyl acetate) to give the product (3.5 g, 68%) as a foamy solid. The solid was dissolved in ethanol (100 mL) and 5 N aqueous sodium hydroxide solution (3.0 mL) was added. The mixture was stirred for 4 h at room temperature. The white precipitate was collected by filtering and washed with 50% ethanol in diethyl ether to give a white solid (3.3 g, 95% (65% overall)) in racemic form as a sodium salt. $^1$H-NMR (500 MHz, $D_2O$) : δ 3.34 (m, 1H), 3.65 (m, 2H), 7.00 (d, 1H), 7.05 (dd, 1H), 7.19 (m, 1H), 7.36 (m, 1H), 7.66 (m, 3H). $^{13}$C-NMR (500 MHz, $D_2O$) : δ 48.9, 55.6, 104.2, 118.5, 121.6, 125.9, 126.0, 127.5, 128.0, 128.6, 135.9, 146.7, 180.0. LC-MS (ESI) calcd for $C_{13}H_{14}N_2O_2$ (M+1) 231.1, obsd. 231.2.

**LeuRS mutant library construction.** A plasmid, pBK-LeuRS, containing Kan$^r$, the ColE1 ori, and multiple cloning sites was used to generate the LeuRS mutant library. L38 and M40 mutations were introduced by quick-change using primers with the desired randomized sequences according to the manufacturer's protocol (Qiagen). Y499, Y500, H537, L538, F541, and A560 mutations were introduced by generating a 900 bp fragment of the LeuRS gene with *Mfe*I and *Asc*I restriction sites at each end by overlap-PCR from primers with the desired randomized sequences. The corresponding 900 bp fragment of the WT-LeuRS gene was then replaced with the randomized fragment. The pBK-LeuRS library plasmid was digested with *Not*I and *EcoR*I and ligated into the corresponding site of the plasmid pA5/L5$_{CUA}$ (*S4*) to yield pEcLRS/L5$_{CUA}$. All constructs and the diversity of the library were confirmed by DNA sequencing. The library was transformed into *S. cerevisiae* Mav203: pGADGAL4 (2TAG).

**Selection of mutant synthetases.** For positive selection, yeast cells ($10^9$ cells) were grown in 50 mL of SD –Leu, –Trp containing 1 mM unnatural amino acid for 12 h at 30 °C. Cells were then harvested by centrifugation and washed twice with 0.9% NaCl. The cells ($10^9$) were plated on SD –Leu, –Trp, –Ura agar plates containing 1 mM unnatural amino acid. After 48 to 60 hours at 30 °C, cells were harvested from the plates and washed twice with 0.9% NaCl. For negative selection, yeast cells ($10^9$ cells) were grown in 50 mL of SD –Leu, –Trp for 12 h at 30 °C. Cells were then harvested by centrifugation and washed twice with 0.9% NaCl. The cells ($10^9$) were plated on SD –Leu, –Trp containing 0.1% 5-FOA. After 48 hours at 30 °C the

cells were harvested from the plates and washed twice with 0.9% NaCl. For the final positive selection, yeast cells ($10^9$ cells) were grown in 50 mL of SD –Leu, –Trp containing 1 mM unnatural amino acid for 12 h at 30 °C. Cells were then harvested by centrifugation and washed twice with 0.9% NaCl. The cells ($10^4$–$10^5$) were plated on SD –Leu, –Trp, –Ura agar plates containing 1 mM unnatural amino acid. After 60 h at 30 °C, 96 individual synthetase clones were selected and each was suspended in 100 µL of 0.9% NaCl in a 96-well plate, and replica-spotted on two SD –Leu, –Trp, –Ura agar plates, one with 1 mM unnatural amino acid and the other without unnatural amino acid. After 48–60 h at 30 °C, clones that grew only on the plate with unnatural amino acid were selected and characterized.

**Expression and purification of the hSOD-33TAG mutant**. Expression was performed with strain SCY4 containing the hSOD expression plasmid pC1SOD-33TAG-$His_6$ and the $tRNA_{CUA}^{Leu5}$/LeuRS encoding plasmid (*S5*). Cultures were grown to saturation in SD –Leu, –Trp at 30 °C and diluted 50-fold into SD –Leu, –Trp containing 1 mM Nap or 0.4 mM Anap. The culture was incubated for 36 h at 30 °C and cells were harvested. Cells were lysed with Y-PER lysis reagent (Pierce) containing complete protease inhibitor cocktail (tablets, EDTA free, Roche) and centrifuged (6000*g*); buffer was exchanged with native purification buffer containing 50 mM $NaH_2PO_4$ (pH 8.0), 200 mM NaCl and 10 mM imidazole. The protein was purified by Ni-NTA affinity chromatography under native conditions according to the manufacturer's protocol (Qiagen).

**Expression and purification of the QBP-160TAG mutant.** The QBP gene was amplified from *E. coli* genomic DNA, digested with *Sal*I and *Bam*HI, and ligated into predigested pHL which was derived from pGFP (*S6*) to express QBP under the ADH1 promotor and terminator. Site-directed mutagenesis was used to introduce the N160TAG mutation into the QBP gene to afford pHL-QBP-160TAG. The Anap-2C gene was amplified by PCR from pEcLRS/$L5_{CUA}$-Anap-2C, digested with *Spe*I and *Xho*I, and ligated into the precut pSNR-LeuRS (*S6*) to afford pSNR-Anap-2C. Expression was performed with yeast strain LWUPF1Δ (*S6*) containing pHL-QBP-160TAG and pSNR-Anap-2C. Cultures were grown to saturation in SD –Leu, –Trp at 30 °C and diluted 50-fold into SD –Leu, –Trp containing 0.4 mM Anap. The culture was incubated for 36 h at 30 °C and cells were harvested. Cells were lysed with Y-

PER lysis reagent (Pierce) containing complete protease inhibitor cocktail (tablets, EDTA free, Roche) and centrifuged; buffer was exchanged with native purification buffer containing 50 mM $NaH_2PO_4$ (pH 8.0), 200 mM NaCl and 10 mM imidazole. The protein expressed was purified by Ni-NTA affinity chromatography under native conditions according to the manufacturer's protocol (Qiagen).

**Fluorescence measurements.** Measurements were performed on an ISS-PC1 photon-counting steady-state spectrofluorometer at room temperature. Fluorescence of Anap at 100 nM (200 μL) in different solvents was measured with excitation at 350 nm. Coumarin 1 was used as a reference to calculate the quantum yield of Anap. Fluorescence of QBP-Anap at 10 nM (200 μL) was measured in D-PBS (GIBCO cat# 14190-144) at different concentrations of L-glutamine (0, 2.5, 7.5, 20, 50 and 100 nM) with excitation at 350 nm. The dissociation constant for QBP-Anap was calculated from fluorescence changes at 430 nm by using the equation, $\Delta F_{obs} = \Delta F_{max}S / (K_d + S)$, where $\Delta F_{obs}$ is the change in fluorescence intensity, $\Delta F_{max}$ is the maximum attainable change in fluorescence intensity, S is the molar concentration of free glutamine, and $K_d$ is the dissociation constant. For QBP-WT (50 nM), intrinsic tryptophan fluorescence changes in different concentrations of L-glutamine (0, 15, 30, 60, 120, 240 and 360 nM) were measured in D-PBS at 339 nm with excitation at 295 nm.

**References**


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**Table S1.** Selected Nap-specific LeuRS mutants.

| LeuRS | frequency | Met40 | Leu41 | Tyr499 | Tyr527 | His537 |
|---|---|---|---|---|---|---|
| Nap-1 | 6/10 | Gly | Pro | Gly | Ala | Thr |
| Nap-2 | 3/10 | Arg | Pro | Ser | Ala | Thr |
| Nap-3 | 1/10 | Gly | Pro | Ile | Ala | Gln |

**Table S2.** Mutated residues for each library. B = C, G, T; K = G, T; M = A, C; N = A, C, G, T; R = A, G; V = A, C, G; W = A, T; Y = C, T.

| LeuRS-WT | Leu 38 | Met 40 | Tyr 499 | Tyr 500 | His 537 | Leu 538 | Phe 541 | Ala 560 |
|---|---|---|---|---|---|---|---|---|
| Lib. 1 | NNK | GGT | GTT | NNK | GAA | NNK | NNK | GNK |
| Lib. 2 | NTT | NGC | RBT | YWT | RMV | WGT | WGT | NTT |

**Figure S1.** Synthesis of unnatural amino acids. Reagents: (a) 2,4-dimethoxybenzylamine, $NaHSO_3$, $H_2O$, 140 °C, 24 h; ethyl 3-bromo-2-(hydroxyimino) propanoate, cesium carbonate, DMF, rt, 1 h, 26%. (b) sodium borohydride, EtOH, rt, 2 h, 96%. (c) Al amalgam, THF/$H_2O$, rt, 2 h, 86%. (d) di-*tert*-butyl dicarbonate, $CH_2Cl_2$, rt, 1 h, 73%. (e) $MnO_2$, acetone, rt, 12 h, 80%. f) trifluoroacetic acid (TFA), $CH_2Cl_2$, rt, 1 h; 1 N NaOH, EtOH, rt, 6 h, 65%. (g) ethyl 3-bromo-2-(hydroxyimino) propanoate, cesium carbonate, DMF, rt, 1 h, 85%. (h) Al amalgam, THF/$H_2O$, rt, 2 h, 80%. (i) 5 N NaOH, EtOH, rt, 6 h, 95%.

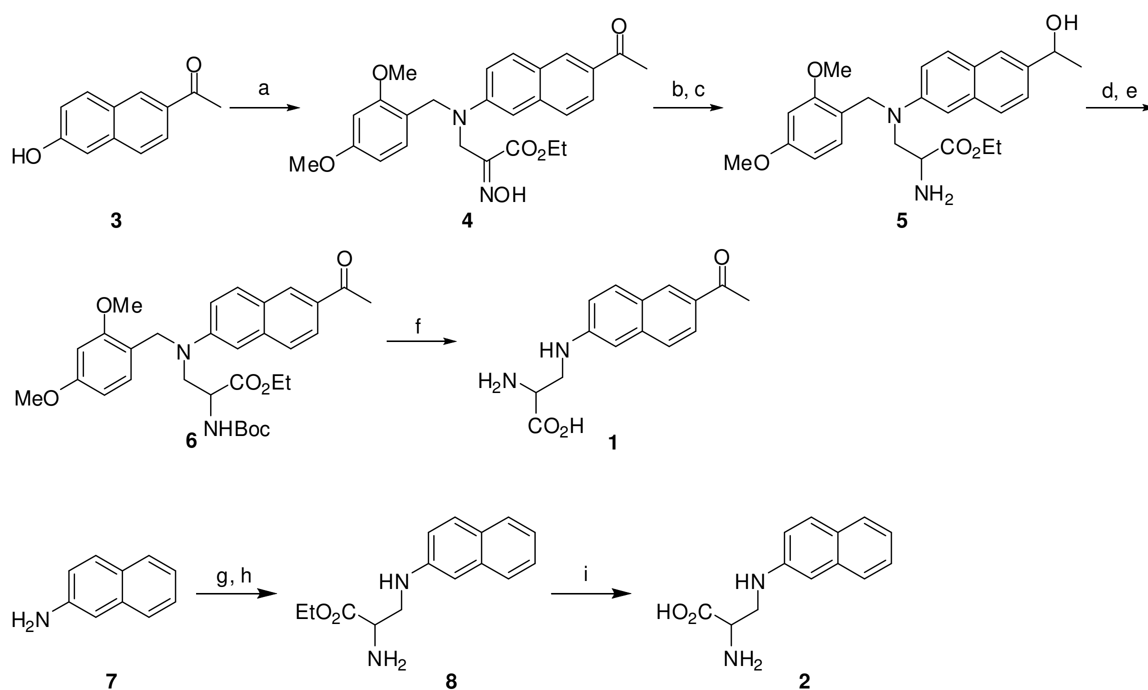

**Figure S2.** Quantum yield measurements. Fluorescence was measured in EtOH with excitation at 360 nm. The quantum yield (0.48) of Anap was calculated relative to the reference quantum yield (0.73) of coumarin 1 (*S7*).

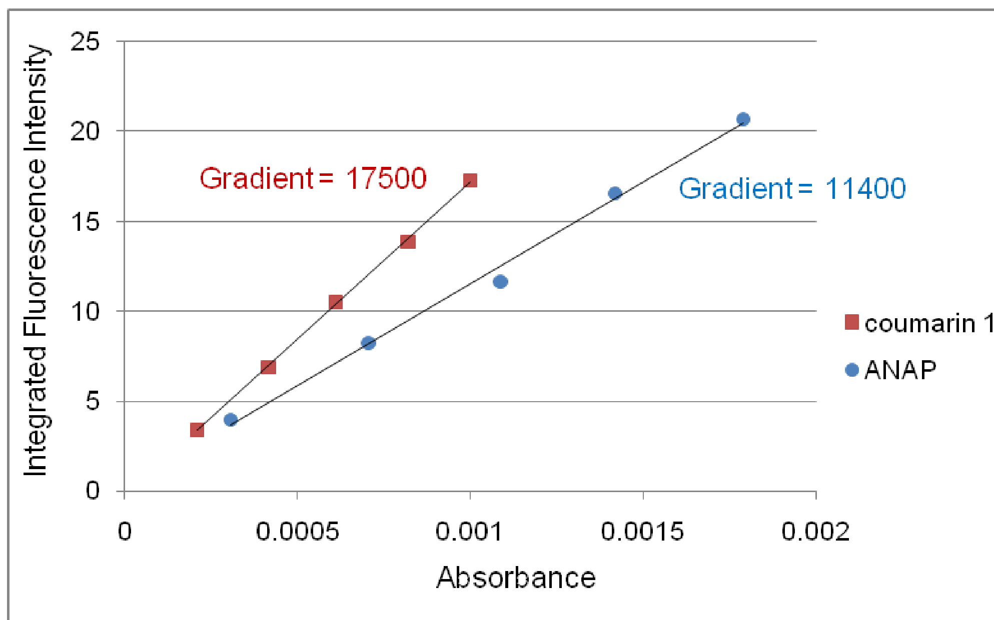

**Figure S3.** Expression of hSOD with an amber codon at 33 position in the presence of the $tRNA_{CUA}^{Leu}$ /Nap-1 pair. (**A**) SDS/PAGE analysis. The gel was stained by GelCode Blue (Pierce). (**B**) MALDI-TOF MS analysis: calculated 16696; observed 16699.

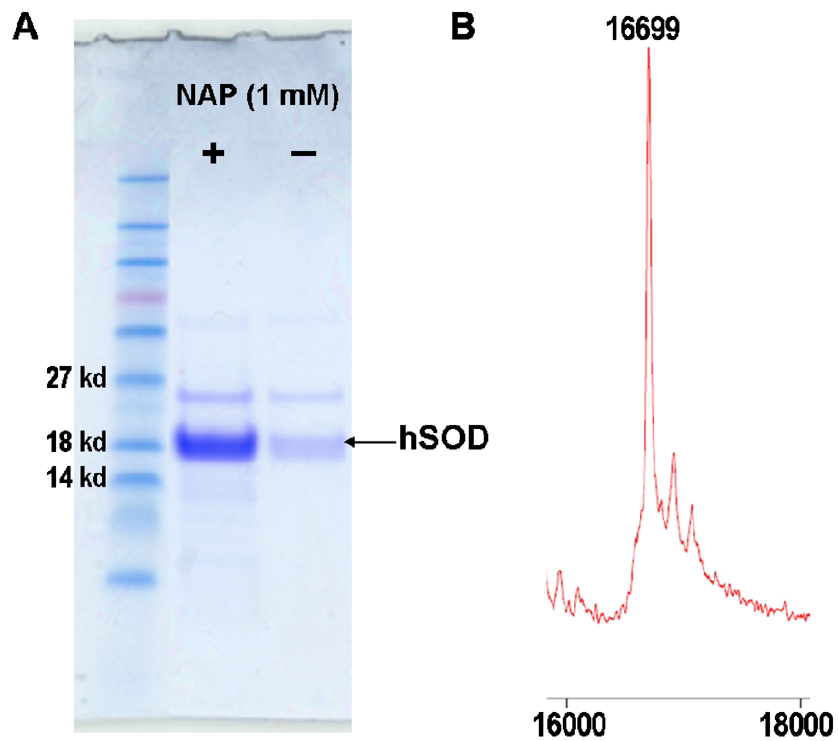

**Figure S4.** ESI-mass spectrometric analysis of QBP-N160Anap and QBP-WT. (**A**) QBP-N160Anap, calculated 25858, observed 25858. (**B**) QBP-WT, calculated 25718, observed 25715.

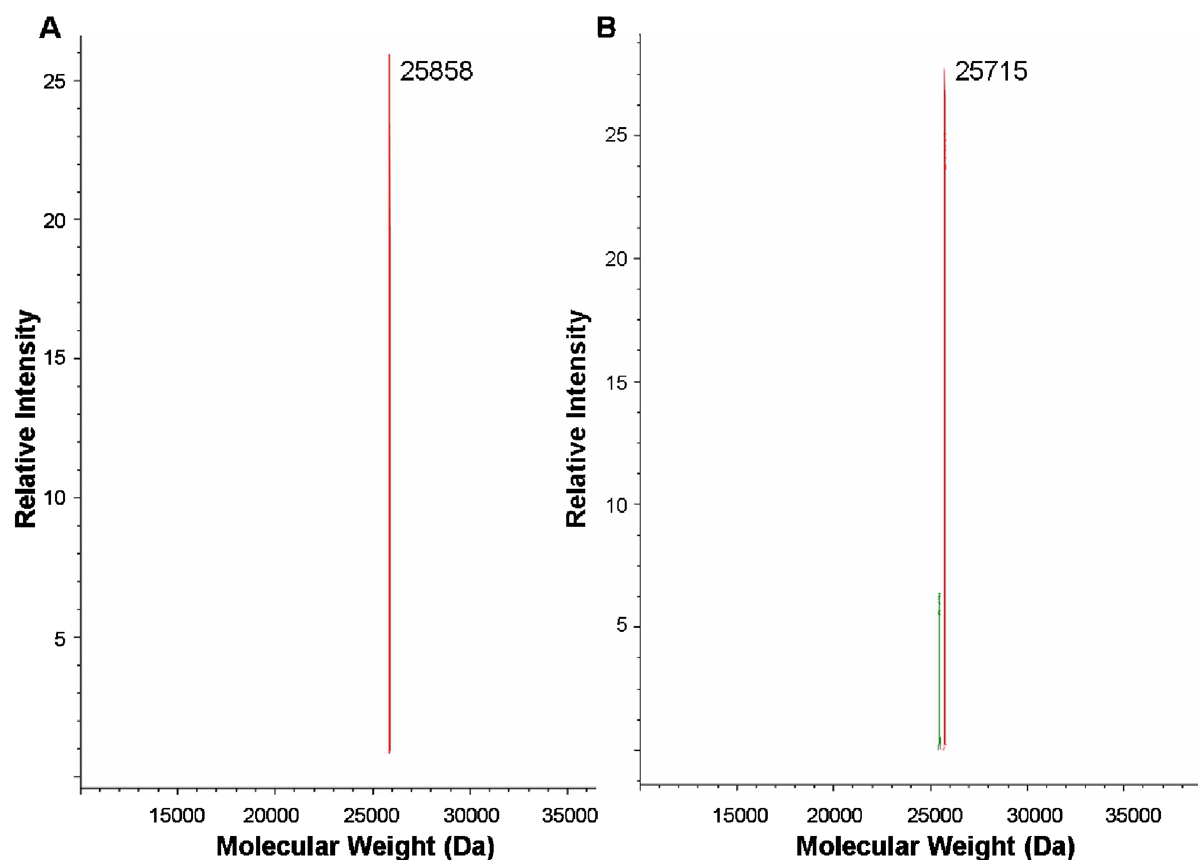

**Fig. S5.** Titration of QBP-WT (50 nM) with different concentrations of glutamine in D-PBS. Fluorescence was measured at 339 nm with excitation at 295 nm.