5-(2-Aminoethyl)-Dithio-2-Nitrobenzoate as a More Base-Stable Alternative to Ellman's Reagent

(Supporting Information)

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Materials. Co(II)-substituted *Bacillus subtilis* LuxS were overexpressed in *Escherichia coli* and purified to near homogeneity as previously described.[1] Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases were from New England Biolabs (Beverly, MA). Talon resin was from Clontech Laboratories (Palo Alto, CA). SRH was enzymatically synthesized from *S*-adenosylhomocysteine (Sigma-Aldrich, St. Louis, MO) using nucleosidase Pfs.[1] All other chemicals were purchased from Sigma-Aldrich.

Cloning, Expression, and Purification of E. coli LuxS. The gene coding for *E. coli* LuxS protein[2] was cloned by using the polymerase chain reaction (PCR) with genomic DNA from BL21(DE3) cells as template and primers 5'-GGGAATTCCATATGCCATTGTTAGATAGCTTCAGAGT-3' and 5'-GGGCTCGAGGATGTGCAGTTCCTGCAACTTCTC-3'. The PCR product was digested with restriction endonucleases *NdeI* and *XhoI* and cloned into the prokaryotic expression vector pET22b(+) (Novagen, WI). This cloning procedure resulted in the addition of a six-histidine tag to the C-terminus of the protein. The identity of the construct was confirmed by DNA sequencing.

E. coli BL21(DE3) cells (4 L) carrying the plasmid DNA were grown in minimal media supplemented with 75 mg/L ampicillin, 0.25% D-glucose, 2 μg/mL thiamin, 1 μg/mL D-biotin, 0.1% $(NH_4)_2SO_4$, and a metal salt mixture (0.5 mM $MgSO_4$, 0.5 μM

[1] Zhu, J.; Dizin, E.; Hu, X.; Wavreille, A.-S.; Park, J.; Pei, D. *Biochemistry* **2003**, 42,4717-4726.
[2] Blattner, F. R.; Plunkett, G. III.; Bloch, C. A.; Perna, N. T.; Burland, V.; Riley, M.; Collado-Vides, J.; Glasner, J. D.; Rode, C. K.; Mayhew, G. F.; Gregor, J.; Davis, N. W.; Kirkpatrick, H. A.; Goeden, M. A.; Rose, D. J.; Mau, B.; Shao, Y. *Science* **1997**, 277, 1453.

H_3BO_3, 0.1 μM $MnCl_2$, 0.5 μM $CaCl_2$, 10 nM $CuSO_4$, 1 nM ammonium molybdate) at 37 °C to an OD_{600} of 0.7. The cells were induced by the addition of 100 μM isopropyl β-D-thiogalactoside and 100 μM $CoCl_2$, and continued to grow at 30 °C for an additional 10 h. Cells were harvested by centrifugation and resuspended in 80 mL of lysis buffer containing 20 mM Tris (pH 8.0), 0.5 M NaCl, 5 mM imidazole, 1% Triton X-100, 0.5% protamine sulfate, and 70 μg/mL chicken egg white lysozyme. The cells were lysed by stirring for 20 min at 4 °C, followed by brief sonication and centrifugation. The supernatant was loaded on a Talon metal affinity column (Clontech, 3.0 x 2.5 cm) equilibrated in 20 mM Tris (pH 8.0), 0.5 M NaCl, and 5 mM imidazole. The column was eluted with the above buffer containing 60 mM imidazole. Fractions containing significant amounts of LuxS protein (as analyzed by SDS-PAGE) were pooled and concentrated in an Amicon apparatus (Millipore). Glycerol was added to a final concentration of 33% (v/v), and the enzyme was quickly frozen in isopropanol dry ice bath and stored at -80 °C. Protein concentration was determined by the Bradford method using bovine serum albumin as standard and corrected by a factor of 0.4 (based on the metal analysis results of *B. subtilis* Co-LuxS).[1]

Synthesis of 5-(2-Aminoethyl)-dithio-2-nitrobenzoate (ADNB). 5-(2-Aminoethyl)-dithio-2-nitrobenzoate was prepared as described by Zhang, Le, and Means.[3] The reaction was performed under an argon atmosphere. NaOH (0.1 g, 2.5 mmol) was dissolved in 10 mL of water. 5,5-Dithiobis(2-nitrobenzoate) (DTNB, 0.990 g, 2.5 mmol) was added. The solution was stirred at room temperature until DTNB was completely dissolved. 2-Mercaptoethylamine/HCl (0.284 g, 2.5 mmol) was added. An additional one equivalent of NaOH (0.1 g, 2.5 mmol) was added. The solution was stirred at room temperature for two hours. The resulting yellow solid was filtered under vacuum and washed with cold water (3 x 5 mL). The isolated product was treated with 5 mL of hot water, allowed to cool to room temperature, filtered, and dried under vacuum to afford 0.34 g of ADNB as a pale yellow solid (50% yield). ^{1}H NMR (400 MHz, D_2O): δ 7.73 (m, 2H, aromatic), 7.50 (m, 2H, aromatic), 3.00 (t, 2H, CH_2, J = 6.8 Hz), 2.71 (t, 2H, CH_2, J = 6.8 Hz).

[3] Zhang, H.; Le, M.; Means, G. E. *Bioorg. Chem.* **1998**, 26, 356-364.

Kinetic Studies. Background hydrolysis of DTNB and ADNB was determined in buffers of various pH values with 150 μM DTNB or ADNB. The release of TNB was continuously monitored at 412 nm in a Perkin-Elmer λ25 UV-VIS spectrophotometer and the pseudo-first-order rate constants were obtained over the first 2 minutes. The buffers used were 50 mM MES for pH 5–6, 50 mM HEPES for pH 7–8, 50 mM CHES for pH 9, and 50 mM CAPS for pH 10–13. All buffers also contained 150 mM NaCl. The reactions of DTNB or ADNB (150 μM) with β-mercaptoethanol (25 μM) were carried out on an SX.18MV stopped-flow reaction analyzer (Applied Photophysics) and the second-order rate constants were calculated from the slopes of the early linear regions of the progress curves.

pH Profile Analysis of LuxS. LuxS assays were carried out in the buffers described above, which also contained S-ribosylhomocysteine (0 to 70 μM) and DTNB (150 μM) or ADNB (240 μM). The reactions were initiated by the addition of LuxS (final 0.4 μM) and monitored at 412 nm in a Perkin-Elmer λ25 UV-VIS spectrophotometer. The initial rates were recorded from the early linear regions of the progress curves and fitted to the Michaelis-Menten equation to obtain the k_{cat} and K_M values.