

SUPPORTING INFORMATION

Novel Heterocyclic Analogs of Firefly Luciferin

Carolyn C. Woodrooffe^{†}, Poncho L. Meisenheimer[†], Dieter H. Klaubert[†], Yumi Kovic[‡], Justin C. Rosenberg[‡], Curran E. Behney[‡], Tara L. Southworth[‡] and Bruce R. Branchini^{†*}*

[†]Promega Biosciences, LLC, San Luis Obispo, California 93433

[‡]Department of Chemistry, Connecticut College, New London, CT 06320

Bruce R. Branchini^{†*}, Department of Chemistry, Connecticut College, New London, CT 06320.

Telephone: 860 439-2479. Fax: 860 439-2477. E-mail: brbra@conncoll.edu

Carolyn C. Woodrooffe^{†*}, Promega Biosciences, LLC, San Luis Obispo, California 93433.

Telephone: 805-544-8524. Fax: 805-543-1531. E-mail: carolyn.woodrooffe@promega.com

TABLE OF CONTENTS:

Materials and Methods	S2 - S3
Figure S1	S4
Analog Syntheses	S5 –S12

MATERIALS AND METHODS

General Experimental

All reagents were purchased from Sigma and used as received. Microwave reactions were carried out on a Biotage Initiator system. Normal-phase silica gel chromatography was carried out on a Combiflash Rf system (Teledyne Isco). Analytical HPLC was performed on an Agilent 1100-1 using a Synergi 50 mm or ACE 50 mm column with appropriate gradients of acetonitrile in water containing 0.1% TFA. Preparative HPLC was performed on a Waters HPLC system with dual-wavelength detection at 254 nm and 360 nm, with linear gradients of acetonitrile in water containing 0.1% TFA or 10 mM NH₄OAc as indicated, at a flow rate of 20 mL/min (1" C18 silica column) or 50 mL/min (2" C18 silica column). ¹H NMR spectra were acquired on a Varian Mercury 300 MHz instrument, and were referenced to internal solvent peaks. Mass spectral data were acquired on an Esquire 4000 in ESI mode. Enantiomeric excess was assessed using a Daicel OD-RH chiral column (150 mm x 4.6 mm) eluting with a gradient of 25->45% MeCN in 0.1% aqueous TFA over 17 min.

pKa Determinations. pKa values were determined from measurements of absorbance changes as a function of pH. For LH₂ and the analogs, the λ_{max} shifted over the pH range of 6.0 to 11.0. The following shifts in λ_{max} were observed for each compound: LH₂, 328 to 383 nm; BoLH₂, 317 to 367 nm; BiLH₂, 318 to 362 nm; BtLH₂, 318 to 362 nm; BfLH₂, 317 to 355 nm and InLH₂, 325 to 357 nm.

Fluorescence Spectroscopy. Fluorescence emission spectra were obtained using a

Horiba Jobin-Yvon *iHR* imaging spectrometer equipped with a liquid N₂ cooled CCD detector with the excitation source set to 330 nm and the excitation and emission slits set to 1 nm. All spectra were corrected for the spectral response of the CCD using a correction curve provided by the manufacturer. Data were collected at 25 °C in a 0.8 ml quartz cuvette over the wavelength range 400–935 nm. Spectra were collected using solutions of LH₂ or substrate analogs using the buffers described above.

Fluorescence quantum yields were measured by preparing a series of solutions of LH₂ or substrate analog with absorption at 370 nm equal to 0.02, 0.04, 0.06, 0.08 and 0.10 in the appropriate buffers described below. Fluorescence emission spectra were obtained as described above, except that the excitation wavelength was 370 nm, and were integrated to provide relative fluorescence intensities. Measurements were made in 50 mM glycylglycine, pH 8.2, 50 mM AMPD, pH 8.5 or 9.1, or 50 mM sodium bicarbonate, pH 11.0. Emission integrals were plotted against absorbance at 370 nm. The slopes of the lines produced by the fluorescence standards, fluorescein in 0.1 M NaOH and quinine sulfate in 0.5 M sulfuric acid, were used to calculate the relative fluorescence quantum yields of LH₂ and the analogs. The fluorescence quantum yields used for fluorescein and quinine sulfate were 0.79 and 0.55, respectively.

Bioluminescence pH Optima. Optimum conditions for bioluminescence for LH₂ and the substrate analogs were determined from luciferase activity assays performed as described in the main text Materials and Methods over a pH range of 6.0 to 9.5. Assays were performed in 25 mM MES, pH 6.0 – 6.7, 50 mM Tris, pH 6.9 – 7.5, 25 mM glycylglycine, pH 7.8 – 8.2, and 50 mM AMPD, pH 8.5 – 9.5. In all assays, the final concentration of Mg-ATP was 2.0 mM and the concentrations of LH₂ or analogs were at least 6 times their K_m values. Activities were corrected for the spectral response of the R928 PMT.

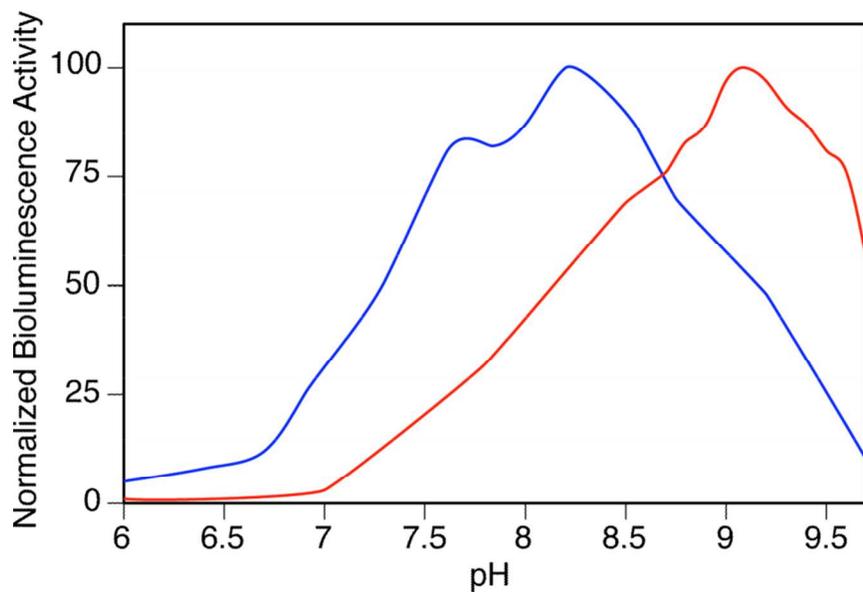
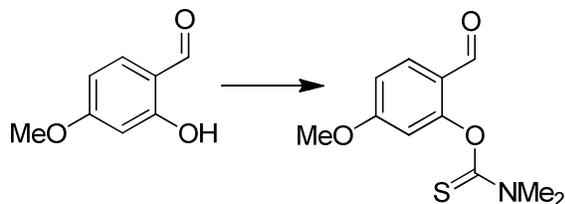


Figure S1. Variation of bioluminescence activity of LH₂ (blue) and BtLH₂ (red) with pH.

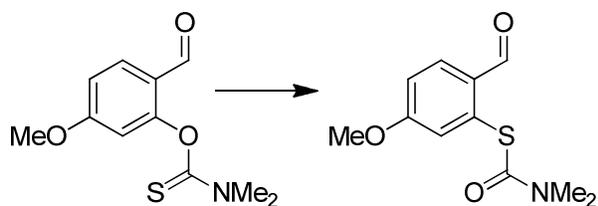
Flash height-based activity assays were performed as described in the main text Materials and Methods and data were corrected for the spectral response of the detector. The flash height intensities are normalized to the maximum value obtained with each substrate. Additional experimental details are described in the Materials and Methods.

Analog Syntheses

Benzothiophene BtLH₂

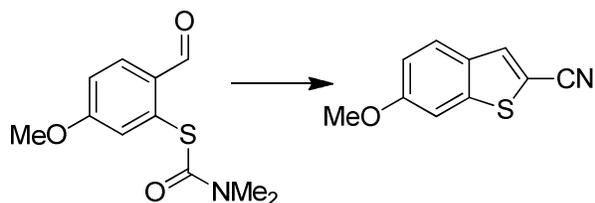


O-(2-Formyl-5-methoxyphenyl) dimethylcarbamothioate. 4-Methoxysalicylaldehyde (10.0 g, 65.7 mmol) was combined with N, N-dimethylaminothiocarbonyl chloride (10.6 g, 85.8 mmol) in 24 mL of dry DMF in an ice bath. DBU (10.4 mL, 69 mmol) was added slowly. The reaction was stirred overnight with concomitant warming to RT. The reaction mixture was then poured into ice water and extracted with 2 x 75 mL EtOAc. The combined organic layers were washed with 2 x 50 mL 1 N HCl, then washed with brine, dried over Na₂SO₄, and evaporated. The resulting material was crystallized from EtOAc layered with heptanes to afford 8.7 g (55%) of light-orange crystals. Additional material could be recovered by silica gel chromatography of the evaporated mother liquor (gradient 0->75% EtOAc in heptanes). ¹H NMR (CD₂Cl₂): 9.92 (s, 1 H); 7.84 (d, 1 H); 6.92 (dd, 1 H); 6.64 (d, 1 H); 3.91 (s, 3 H); 3.45 (s, 3 H); 3.40 (s, 3 H). MS: Calcd for M+H 240.1, found 240.1.



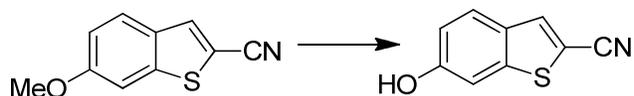
S-(2-Formyl-5-methoxyphenyl) dimethylcarbamothioate. A 2.20 g (9.2 mmol) portion of O-(2-formyl-5-methoxyphenyl) dimethylcarbamothioate was dissolved in 20 mL of dry toluene in a microwave tube. The solution was heated for 8 h at 180 C. Two identical reactions were combined, adsorbed onto Celite, and purified by silica gel chromatography (25->75 % EtOAc in

heptanes). The product was obtained as 2.48 g (56% yield) of a golden-brown oil that began to crystallize on standing. $^1\text{H NMR}$ (CD_2Cl_2): 10.22 (s, 1 H); 7.97 (d, 1 H); 7.03-7.12 (m, 2 H); 3.90 (s, 3 H); 3.15 (br s, 3 H); 3.01 (br s, 3 H). MS: Calcd for $\text{M}+\text{Na}$: 262.1, found 262.1.



6-Methoxybenzo[b]thiophene-2-carbonitrile. S-(2-Formyl-5-methoxyphenyl)

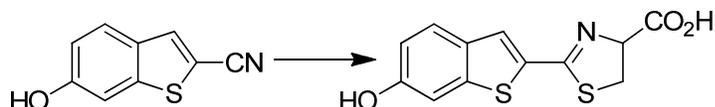
dimethylcarbamothioate (2.07 g, 8.65 mmol) was stirred in 25 mL of water with 8.2 g of NaOH and the suspension was heated to reflux for 3 h. The orange reaction solution was cooled, poured into 100 mL of ice water, washed with Et_2O , and acidified with conc HCl. The resulting suspension was extracted with 3 x 50 mL EtOAc, dried over Na_2SO_4 , and evaporated to afford 1.46 g (quantitative) of a light brown liquid. The intermediate thiophenol ($^1\text{H NMR}$ (CD_2Cl_2): 9.88 (s, 1 H); 7.67 (d, 1 H); 6.82-6.88 (m, 2 H); 6.07 (s, 1 H); 3.88 (s, 3 H). MS: Calcd for $\text{M}+\text{H}$ 169.0, found 169.1) was combined with K_2CO_3 (1.39 g, 10 mmol) and chloroacetonitrile (640 μL , 10 mmol) in 50 mL of dry THF and heated to reflux overnight. The reaction was cooled, filtered, and then adsorbed onto Celite for silica gel chromatography (0- \rightarrow 50% EtOAc in heptanes) to afford 940 mg (57%, 2 steps) of a light yellow crystalline solid. $^1\text{H NMR}$ (CD_2Cl_2): 7.75 (s, 1 H); 7.69 (d, 1 H); 7.22 (d, 1 H); 7.01 (dd, 1 H); 3.79 (s, 3 H). MS: Calcd for $\text{M}+\text{H}$: 190.0, found 190.0.



6-Hydroxybenzo[b]thiophene-2-carbonitrile. 6-Methoxybenzo[b]thiophene-2-carbonitrile

(150 mg, 0.79 mmol) was combined with pyridinium hydrochloride (3.0 g, 26 mmol) in a

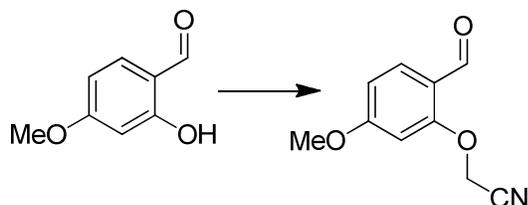
microwave tube and irradiated at 220 C for 30 min. The resulting solid was taken up in EtOAc and 1 N HCl and the layers separated; the aqueous layer was extracted with 2 x EtOAc and the combined organics were washed with brine and dried over Na₂SO₄. Silica gel chromatography 0->50% EtOAc afforded 93 mg (68%) of a fluffy white solid. ¹H NMR (CD₂Cl₂): 7.84 (s, 1 H); 7.79 (d, 1 H); 7.32 (d, 1 H); 7.04 (dd, 1 H). MS: Calcd for (M-H) 174.0, found 174.0.



2-(6-Hydroxybenzo[b]thiophen-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (3-

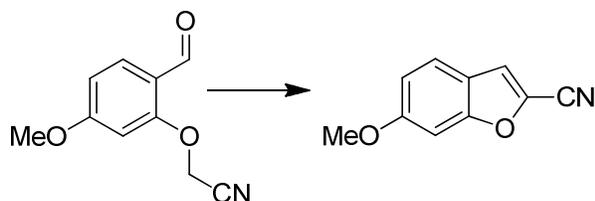
deazaluciferin, BtLH₂). 6-Hydroxybenzo[b]thiophene-2-carbonitrile (80 mg, 0.46 mmol) was combined with D-cysteine hydrochloride hydrate (160 mg, 0.91 mmol) and K₂CO₃ (160 mg, 1.16 mmol) in water/acetonitrile. The reaction was monitored by HPLC. Upon completion, the reaction was neutralized with acetic acid and purified by preparative HPLC using a gradient of 2->100% MeCN in 10 mM NH₄OAc. The solvents were removed under reduced pressure to afford 107 mg (84%) of a creamy solid. ¹H NMR (DMSO - d₆): 7.72 (d, 1 H); 7.59 (s, 1 H); 7.28 (d, 1 H); 6.85 (dd, 1 H); 4.90 (dd, 1 H); 4.68 (dd, 1 H); 4.55 (dd, 1 H). MS: Calcd for (M+H) 280.0, found 280.0. HPLC (319 nm): 100% pure.

Benzofuran BfLH₂

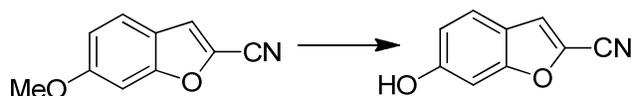


2-(2-Formyl-5-methoxyphenoxy)acetonitrile. 4-Methoxy-2-salicylaldehyde (2.42 g, 15.9 mmol) was combined with bromoacetonitrile (1.34 mL, 2.42 g, 20.2 mmol) and potassium carbonate (2.20 g, 15.9 mmol) in 24 mL of dry THF and heated to reflux overnight. The reaction

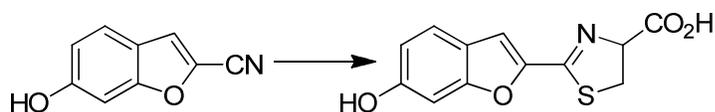
mixture was filtered, the filter cake was washed twice with THF, and the resulting solution was adsorbed onto Celite. Chromatographic purification on silica eluting with 0->50% EtOAc in heptanes afforded 2.36 g (77.8%) of the desired product. ¹H NMR (CD₂Cl₂): 10.25 (s, 1 H); 7.87 (d, 1 H); 6.74 (dd, 1 H); 6.57 (d, 1 H); 4.92 (s, 2 H); 3.92 (s, 3 H). MS: Calcd for M + Na 214.1, found 214.1.



6-Methoxybenzofuran-2-carbonitrile. 2-(2-formyl-5-methoxyphenoxy)acetonitrile (1.00 g, 5.2 mmol) was combined with K₂CO₃ (1.04 g, 7.5 mmol) in 10 mL of DMF and heated to 100 C for 1 h. The reaction was cooled to RT and filtered. The filtrand was washed with DCM. The combined filtrates were washed with 2 x 50 mL H₂O, dried over Na₂SO₄, and evaporated. The residue was purified by silica gel chromatography using a gradient of 0->10% MeOH in DCM. 500 mg (50.5%) of the desired benzofuran was isolated. ¹H NMR (CD₂Cl₂): 7.58 (d, 1 H); 7.45 (s, 1 H); 7.07 (d, 1 H); 7.0 (m, 1 H), 3.9 (s, 3 H). MS: Calcd for M+H 174.1; found 174.5.



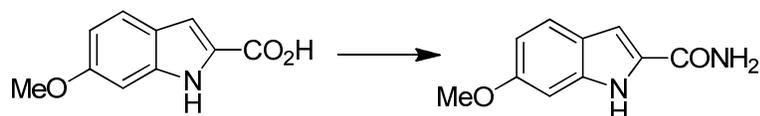
6-Hydroxybenzofuran-2-carbonitrile. 5-methoxybenzofuran-2-carbonitrile (100 mg, 0.58 mmol) was suspended in 1.70 g of solid pyridinium hydrochloride (14.7 mmol) and heated at 220 C for 30 min. After cooling to RT, the reaction was partitioned between DCM and 1 N HCl, and the organic layer was dried over Na₂SO₄ and evaporated to afford 70 mg of crude material (76%). ¹H NMR (CD₂Cl₂): 7.57 (d, 1 H); 7.44 (s, 1 H); 7.04 (d, 1H); 6.95 (m, 1 H). MS: Calcd for M-H 158.0, found 157.9.



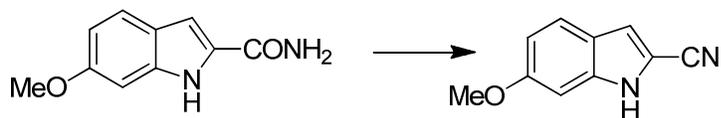
2-(6-Hydroxybenzofuran-2-yl)-4,5-dihydrothiazole-4-carboxylic acid. 5-

Hydroxybenzofuran-2-carbonitrile (70 mg, 0.44 mmol) was stirred in a water/MeCN mix with 155 mg of D-cysteine hydrochloride hydrate (0.88 mmol) and K_2CO_3 (155 mg, 1.1 mmol). The reaction was monitored by analytical HPLC. Upon completion, the reaction mixture was acidified slightly by the addition of AcOH, filtered, and purified by preparative HPLC using a gradient of 2- \rightarrow 50% MeCN in 10 mM NH_4OAc to yield 60 mg of the desired product (52%). 1H NMR (DMSO - d_6): 7.47 (d, 1 H); 7.24 (s, 1 H); 6.96 (d, 1 H); 6.80 (dd, 1 H); 4.88 (dd, 1 H); 3.63 (dd, 1 H); 3.48 (dd, 1H). MS: Calcd for M+H 264.0, found 264.1. HPLC (330 nm): 98.3% pure.

6-Indole InLH₂



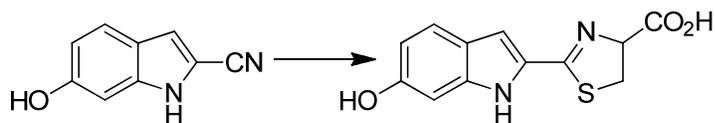
6-Methoxyindole-2-carboxamide. 6-Methoxyindole-2-carboxylic acid (2.10 g, 11 mmol) was suspended in 60 mL of dichloroethane. DMF (3 drops) was added, and then thionyl chloride (3.0 mL, 4.92 g, 41.3 mmol) was added cautiously. The reaction was heated in a 68 C oil bath for 1 h, then cooled and poured into a vigorously stirred mixture of ammonium hydroxide and ice. The organic layer was separated and evaporated and the residue was recombined with the aqueous layer and vigorously stirred. Filtration afforded a creamy crystalline material, which was washed with water and dried to give 1.90 g (91%) of the desired amide. 1H NMR (DMSO - d_6): 7.80 (br s, < 1 H); 7.42-7.48 (m, 1 H); 7.19 (br s, < 1 H); 7.02 (s, 1 H); 6.83-85 (m, 1 H); 6.62-6.70 (m, 1 H); 3.88 (s, 1 H); 3.75 (s, 3 H). MS: Calcd for M+H 191.1, found 191.1.



6-Methoxyindole-2-carbonitrile. 6-Methoxyindole-2-carboxamide (1.84 g, 9.7 mmol) was suspended in 20 mL of DCE and POCl_3 (2.65 mL, 4.44 g, 29 mmol) was added. The reaction was stirred in a 68 C oil bath for 1 h, after which the temperature was increased to 78 C for an additional hour. The reaction was cooled and poured into water; the product was extracted with a mixture of DCM and EtOAc, and the combined organic layers were dried over Na_2SO_4 and adsorbed onto Celite. Silica gel chromatography eluting with a gradient of 0->50% EtOAc in heptanes afforded 1.27 g (76%) of the nitrile as slightly yellow crystals. ^1H NMR (DMSO - d_6): 7.53 (s, 1 H); 7.34-7.36 (m, 1 H); 6.85-6.87 (m, 1 H); 6.77-6.82 (m, 1 H); 3.78 (s, 3 H); 3.30 (s, 1 H). MS: Calcd for M-H 171.1, found 170.9.



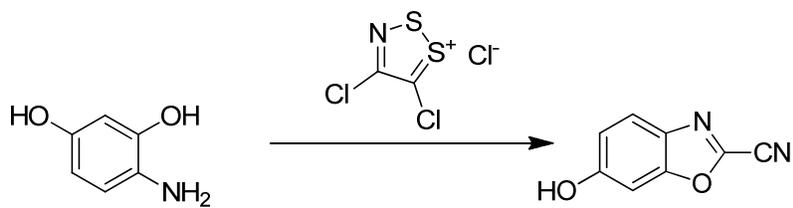
6-Hydroxyindole-2-carbonitrile. 6-Methoxyindole-2-carbonitrile (300 mg, 1.7 mmol) was suspended in 3.0 g (26 mmol) of solid pyridinium hydrochloride and heated at 220 C for 30 min using microwave irradiation. The reaction was partitioned between EtOAc and 1 N HCl; the combined organic layers were washed with brine and adsorbed onto Celite. Silica gel chromatography with a gradient of 0->70% EtOAc in heptanes yielded 172 mg (63%) of a white crystalline solid. ^1H NMR (DMSO - d_6): 11.87 (br s, < 1 H); 9.52 (s, 1 H); 7.43 (d, 1 H); 7.19 (s, 1 H); 6.7-6.73 (m, 1 H); 6.74-6.79 (m, 1 H). MS: Calcd for M-H 157.1, found 157.0.



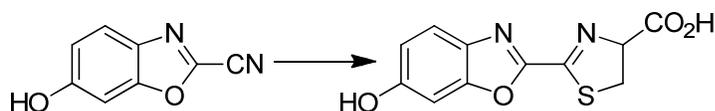
2-(6-hydroxy-1H-indol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (6-hydroxyindole

luciferin, InLH₂) 6-Hydroxyindole-2-carbonitrile (100 mg, 0.63 mmol) was stirred in MeCN and treated with an aqueous solution of D-cysteine hydrochloride hydrate (222 mg, 1.3 mmol) and K₂CO₃ (272 mg, 2.0 mmol). Upon completion of the reaction as judged by HPLC, the product was isolated by preparative HPLC using a gradient of 5->40% MeCN in 10 mM NH₄OAc. ¹H NMR (DMSO - d₆): 7.33 (dd, 1 H); 6.76-6.81 (m, 1 H); 6.64 (d, 1 H); 6.55 (dd, 1 H); 4.88 (dd, 1 H); 3.59 (dd, 1 H); 3.45 (dd, 1 H). MS: Calcd for M+H 263.0, found 263.1. HPLC (330 nm, 360 nm): 100% pure.

Benzoxazole luciferin BoLH₂



6-Hydroxybenzoxazole-2-carbonitrile. To a 20-mL microwave vial was added 4-aminoresorcinol (1.00 g, 8 mmol) and 15 mL of THF. The vial was cooled in an ice bath and 1.67 g (8 mmol) of Appel's salt was added. The reaction was removed from the cooling bath and stirred for 15 min at RT, then heated to 130 C for 15 min. After partitioning between 100 mL of EtOAc and 50 mL of H₂O, the organic layer was washed with 1 x 30 mL H₂O and 1 x 30 mL brine, then dried over Na₂SO₄, adsorbed onto 20 g of Celite, and purified by silica chromatography using a gradient of 0 ->100% EtOAc in heptanes. 155 mg of the title compound was obtained (12% yield). ¹H NMR (DMSO - d₆): 10.5 (s, 1 H); 7.75(d, 1 H); 7.18 (d, 1 H); 7.04 (dd, 1 H). MS: Calcd for (M+H) 161.0, found 161.1.



2-(6-hydroxybenzo[d]oxazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid. D-Cysteine hydrochloride hydrate (239 mg, 1.36 mmol) was dissolved in 1 mL of water and the pH was adjusted to 7 with 1 M NaOH. A solution of 6-hydroxybenzoxazole-2-carbonitrile (145 mg, 0.91 mmol) in 2 mL of DMF was added to the cold neutral solution of D-cysteine. After stirring for 15 min, the reaction was diluted in 10 mL of aqueous MeCN, filtered, and the product was isolated by preparative HPLC eluting with a gradient of 0->100% MeCN in 0.1% TFA. The appropriate fractions were combined and concentrated under reduced pressure, then lyophilized to afford 150 mg (63% yield) of a yellow solid. A 40 mg portion of the solid was purified further by preparative HPLC eluting with a gradient of 0->50% MeCN in 20 mM NH₄OAc to yield 17 mg of solid material. ¹H NMR (CD₃CN): 7.68 (d, 1 H); 7.19 (d, 1 H); 6.99 (dd, 1 H); 5.46 (t, 1 H); 3.77 (d, 2 H). MS: Calcd for (M+H) 265.0, found 265.0. HPLC (330 nm) 95.1% pure.