SUPPORTING INFORMATION

Yellow-Green and Red Firefly Bioluminescence from 5,5-Dimethyloxyluciferin

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EXPERIMENTAL SECTION

Materials and Methods. Reactions were carried out in oven dried glassware, unless otherwise noted. All chemicals and solvents were reagent grade and used as received except where otherwise indicated. D-penicillamine, pyridine-HCl, and anhydrous dimethyl sulfoxide (DMSO) were purchased from Aldrich. D-Firefly luciferin (LH_2) was obtained from Biosynth and adenosine-5´-monophosphate from Sigma. Pyridine-HCl was dried overnight *in vacuo* over phosphorus pentoxide prior to use. The following were prepared by previously reported procedures: 2-cyano-6-hydroxybenzothiazole[1], 5,5-dimethyloxyluciferin[2], and luciferyl-O-adenosine-5´-monophosphate[3] (LH_2-AMP). *Photinus pyralis* luciferase containing the additional N-terminal peptide Gly-Pro-Leu-Gly-Ser- (Ppy) was prepared as described[4] previously. Unless otherwise specified, all salts and stabilizing reagents were removed from preparations of luciferases by exchanging the storage buffers into 0.1 M sodium phosphate buffer, pH 8.0 using gel filtration chromatography (PD-10 columns, Pharmacia). Melting points were determined with a Mel-Temp II apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer model 16PC FT-IR spectrometer. Proton and carbon-13 NMR spectra were recorded on a Bruker AC-250 NMR spectrometer. Chemical shifts (δ) are reported in ppm relative to internal tetramethylsilane (δ = 0.00). Optical rotations were obtained with a Jasco P-1010 polarimeter. Mass spectra were measured with either a tandem HPLC-electrospray ionization system consisting of a Perkin-Elmer Series 200 HPLC and a Sciex ABI150A mass spectrometer or a Micromass Ultima triple quadrapole attached to a Perkin-Elmer 140D Solvent Delivery system. High-performance liquid chromatography (HPLC) was performed with a LDC/Milton-Roy CM4000 system. Detailed procedures including descriptions of equipment used to determine bioluminescence activity of luciferases by flash height- and integration-based light assays have been previously reported[4,5,6,7]. Additionally, these references[4-7] include the procedures, equipment and methods previously used to obtain the following: fluorescence emission spectra; steady-state kinetic constants for LH_2, LH_2-AMP, and ATP-Mg; pH optima; and total protein.

Synthesis of 5,5-Dimethylluciferin (D-DiMeLH$_2$). This procedure was based on the method of White[8] for the preparation of D,L-5,5-dimethylluciferin. Under a nitrogen atmosphere, a solution of D-penicillamine (94.0 mg, 0.63 mmol) in 1.4 mL of deoxygenated (nitrogen bubbling for 15 min) water (adjusted to pH 8.0 (litmus) with 0.3N NaOH) was added to 2-cyano-6-hydroxybenzothiazole (102.0 mg, 0.58 mmol) dissolved in deoxygenated (nitrogen bubbling for 15 min) methanol (4.2 mL). After the resulting yellow mixture had stirred at room temp for 2 h, water (20 mL) was added and the pH of the mixture was adjusted to 8.5 (litmus). The solution was extracted with ether (2 x 20 mL) and then with ethyl acetate (2 x 20 mL) to remove neutral compounds. The aqueous solution was cooled (ice bath) and acidified to pH 1 (litmus) by drop-wise addition of 5% HCl. A yellow precipitate formed that was collected by vacuum filtration, washed with cold water, and dried *in vacuo* providing 112.0 mg crude product (0.63 mmol, 64% yield). Recrystallization from methanol-water produced 62.0 mg (0.20 mmol, 35% yield) of pale yellow needle-like crystalline product: mp 187.8 – 188.2 °C, $[\alpha]^{22}_{D}$ -31.10° (c 0.002 DMSO); IR (KBr) 2972 (w), 1728 (s), 1569 (s), 1481 (s), 1224 (s), 1068 (s), 866 (s) cm^{-1}; ^{1}H NMR (250 MHz, DMSO – d_6) δ 13.14 (br s, 1H), 10.20 (br s, 1H), 7.92 (d, J = 8.9 Hz, 1H), 7.43 (d, J = 2.4 Hz, 1H), 7.04 (dd, J = 8.9, 2.4 Hz, 1H), 4.95 (s, 1H), 1.71 (s, 3H), 1.44 (s, 3H); ^{13}C NMR (62.9 MHz, DMSO – d_6) δ 169.5, 163.5, 157.3, 146.2, 137.1, 124.8, 117.1, 106.8, 85.5, 60.3, 28.5, 25.7; mass spectrum *m/z* 309 [(M + H)$^+$; calcd for $C_{13}H_{12}N_2O_3S_2$, 309.10].

Preparation of D-5,5-Dimethylluciferyl-O-adenosine-5´-monophosphate (D-DiMeLH$_2$-AMP). The synthesis of D-DiMeLH$_2$-AMP was similar to that previously described[3] for LH_2-AMP. Under an argon atmosphere, a solution of 100 mg (0.485 mmol) of dicyclohexylcarbodiimide in 0.8 mL DMSO was added to a solution of D-DiMeLH$_2$ (5 mg, 0.016 mmol) and adenosine-5´-monophosphate (15 mg, 0.043 mmol), in 1 ml dry DMSO. The reaction mixture was stirred vigorously for 10 min at room temp and acetone (5 mL) was added to quench the reaction. A white precipitate formed and was collected by centrifugation, washed twice with ice cold acetone (3 mL) and extracted with 10 mM sodium acetate, pH 4.5, containing 40 mM sodium chloride (2 x 0.75 mL). D-DiMeLH$_2$-AMP was isolated from the pooled extracts by RP-HPLC on a C18 column (Microsorb MV, 4.5 x 250 mm), at a flow rate of 1.0 mL/ min, using 25 mM ammonium acetate, pH 4.5, containing linear gradients of CH_3CN: 10% initially, 10% after 15 min, 45% after 35 min. D-DiMeLH$_2$-AMP eluted in ~ 35% CH_3CN and was stored in 25 mM ammonium acetate: CH_3CN, 65:35, v/v at –70 °C and was stable for ~ 4 months on storage. Prior to use, the CH_3CN was evaporated under a gentle stream of argon. UV (25 mM ammonium acetate: CH_3CN, 65:35, v/v) λ_{max} 336 nm (ε = 16,000) and 260 nm (ε = 16,000); mass spectrum *m/z* 638.1 [(M + H)$^+$; calcd for $C_{23}H_{24}N_7O_9PS_2$, 638.5].

Subcloning, expression and purification of *Pyrophorus plagiophthalamus* luciferase (PplGR). The pSx (Tac) vector (Promega) containing the gene for the wild-type green light emitting isozyme of *Pyrophorus plagiophthalamus* luciferase (PplGR) required modification to produce a template with the correct reading frame required for subcloning into the pGex-6p-2 fusion vector. The Chameleon (Stratagene) double-stranded mutagenesis kit was used to realign the cDNA reading frame by removing one base pair and to introduce an *Eco*RV endonuclease site using the following primer (Life Technologies): 5´-CACACAG**GATATC**GGATC-CATGATGAAGAG-3´(underline represents the restriction site, bold indicates the silent changes to create the site and the dash (-) is the position of the deleted base pair). The selection primer used to change the nonessential *Sca*I endonuclease site was: 5´-GTGACTGGTG**ACTCCT**CAACCAAG-3´ (underline represents the restriction site and bold indicates the mutated codons). The DNA insert was isolated from the modified pSx vector by digestion with *Bam*HI and *Xho*I, and sub-cloned directly into the pGex-6p-2 expression vector (Amersham-Pharmacia). The modified vector was transformed into the *E. coli* strain BL21.

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[1] Seto, S.; Ogura, K.; Nishiyama, Y. *Bull. Chem. Soc. Japan* **1963**, 36, 331-333.

[2] White, E. H.; Rapaport, E.; Seliger, H. H.; McElroy, W. D. *Bioorg. Chem.* **1971**, *1*, 92-122.

[3] Branchini, B. R.; Magyar, R. A.; Murtiashaw, M. H.; Portier, N. C. *Biochemistry* **2001**, *40*, 2410-2418.

[4] Branchini, B. R.; Magyar, R. A.; Murtiashaw, M. H.; Anderson, S. M.; Zimmer, M. *Biochemistry* **1998**, *37*, 15311-15319.

[5] Thompson, J. F.; Geoghegan, K. F.; Lloyd, D. B.; Lanzetti, A. J.; Magyar, R. A.; Anderson, S. A.; Branchini, B. R. *J. Biol. Chem.* **1997**, *272*, 18766-18771.

[6] Branchini, B. R.; Magyar, R. A.; Murtiashaw, M. H.; Anderson, S. M.; Helgerson, L. C.; Zimmer, M. *Biochemistry* **1999**, *38*, 13223-13230.

[7] Branchini, B. R.; Murtiashaw, M. H.; Magyar, R. A.; Anderson, S. M. *Biochemistry* **2000**, *39*, 5433-5440.

[8] White, E. H.; Wörther, H.; Field, G. F.; McElroy, W. D. *J. Org. Chem.* **1965**, 30, 2344-2348.

The glutathione-S-transferase (GST) fusion construct of PplGR, was expressed in *E. coli* strain BL21. A culture (250 mL) was grown in a 1 L flask at 37 ˚C in LB medium supplemented with 100 μg/mL ampicillin to mid log phase (A_{600} = 0.6-0.9), induced with 0.1 mM IPTG, and incubated at 25 ˚C for 23 h. The cells were harvested by centrifugation at 4 ˚C. The cell pellet was frozen and kept at -70 ˚C for 30 min and resuspended in 25 mL of a solution of 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3) containing 0.1 mM phenylmethylsulfonyl fluoride and 5 mM DTT. Lysozyme (0.05 volume of 10 mg/mL) was added and the cells were lysed by sonication. The lysate was treated with RNase (10 μg/mL) and DNaseI (5 μg/mL); Triton X-100 (2% final volume) was added and the whole-cell extract was isolated by centrifugation at 20,000g for 1 h. The luciferase was purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions as described[4] previously. The PplGR enzyme (1.2 mg) was stored at 4 ˚C in 1 mL of a buffer consisting of [50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA and 5 mM DTT] containing 0.8 M ammonium sulfate and 2% glycerol. The luciferase contained the additional N-terminal peptide Gly-Pro-Leu-Gly-Ser- and had a molecular mass of 61,104 Da, which is within ± 1 mass units of the calculated value.

Product analyses of reactions of *Photinus pyralis* (Ppy) and *Pyrophorus plagiophthalamus* (PplGR) luciferases with D-DiMeLH$_2$-AMP. The product analyses were carried out in triplicate. Ppy, (16.3 nmol) and D-DiMeLH$_2$-AMP (6.0 nmol) were incubated in 0.1 M sodium phosphate buffer, pH 8.6 (0.5 mL final volume) for 30 min at room temp and bioluminescence was monitored. After light emission ceased, solid urea (final [urea] = 8 M) and the internal standard 4-nitroaniline (6.0 nmol) were added. The reaction mixture was analyzed by RP-HPLC using a C-18 column (Rainin, 10 x 250 mm) eluted at 3 mL/min, with 25 mM ammonium acetate, pH 4.5, containing linear gradients of CH_3CN: 10% initially, 10% after 5 min, 20% after 10 min, 40% after 30 min, 50% after 35 min, 50% at 45 min. Authentic standards of D-DiMeLH$_2$-AMP, D-DiMeLH$_2$, 4-nitroaniline and 5,5-dimethyloxyluciferin eluted at 21, 24, 29 and 41.5 min, respectively. The spent reaction mixture contained unreacted D-DiMeLH$_2$-AMP (0.6 nmol) and products D-DiMeLH$_2$ (4.5 nmol) and 5,5-dimethyloxyluciferin (0.9 nmol). Chromatography was monitored at 350 nm with a Spectromonitor 3000 variable wavelength detector. The identity of the products, based initially on retention times, was confirmed by fluorescence emission spectroscopy and mass spectral analysis. D-DiMeLH$_2$: fluorescence, excitation λ = 380 nm, emission λ_{max} = 537 nm; mass spectrum, *m/z* 309; [$(M + H)^+$; calcd for $C_{13}H_{12}N_2O_3S_2$, 309.3] and 5,5-dimethyloxyluciferin: fluorescence, excitation λ = 390 nm, emission λ_{max} = 630 nm; mass spectrum, *m/z* 279; [$(M+ H)^+$; calcd for $C_{12}H_{10}N_2O_2S_2$, 279.4]. A control experiment to determine nonspecific hydrolysis of D-DiMeLH$_2$-AMP was conducted exactly as described above except that heat inactivated Ppy was used. No bioluminescence was observed and the product analysis (conducted as described above) indicated that only unreacted D-DiMeLH$_2$-AMP (1.8 nmol) and D-DiMeLH$_2$ (4.2 nmol) were present. Based on the amount of D-DiMeLH$_2$-AMP that was not expected to hydrolyze (1.8 - 0.6 = 1.2 nmol), active Ppy produced D-DiMeLH$_2$ (4.5 - 4.2 = 0.3 nmol, 25%) and 5,5-dimethyloxyluciferin (0.9 nmol, 75%). Based on the assumption that D-DiMeLH$_2$ was produced by an enzyme-catalyzed dark reaction, a quantitative yield of 5,5-dimethyloxyluciferin was obtained for the bioluminescence process.

A similar study with PplGR and DiMeLH$_2$-AMP was conducted as described above except that the total incubation time was 10 min and 5.0 nmol of DiMeLH$_2$-AMP was reacted. The spent reaction mixture contained unreacted D-DiMeLH$_2$-AMP (2.3 nmol) and products D-DiMeLH$_2$ (2.1 nmol) and 5,5-dimethyloxyluciferin (0.55 nmol). Based on the amount of D-DiMeLH$_2$-AMP that was not expected to hydrolyze (3.8 - 2.3 = 1.5 nmol), active PplGR produced D-DiMeLH$_2$ (2.1 - 1.2 = 0.9 nmol, 60%) and 5,5-dimethyloxyluciferin (0.55 nmol, 37%). Based on the assumption that D-DiMeLH$_2$ was produced by an enzyme-catalyzed dark reaction, an approximately quantitative yield of 5,5-dimethyloxyluciferin was obtained for the bioluminescence process.

Bioluminescence emission spectra. Bioluminescence emission spectra were obtained using a Perkin-Elmer LS55 luminescence spectrometer operated in the "bioluminescence" mode. Data were collected in the wavelength range 480 - 680 nm in a 1 mL optical glass fluorescence cuvette. Gate and delay times, detector voltage, scan rate, and slit width were adjusted to optimize instrument response. Data were corrected for the spectral response of the R928 photomultiplier tube using the FL WinLab software. Bioluminescence emission spectra were obtained with final concentrations of Ppy (0.1-0.3 μM) and PplGR (0.2-0.5 μM) and the following substrates (final concentrations in 0.6 mL volume): (1) 70 μM LH$_2$ plus 2 mM Mg-ATP (a) in 0.1 M sodium phosphate buffer, pH 8.6; and (b) in 0.05 M MES buffer, pH 6.0; (2) 0.35 μM D-DiMeLH$_2$-AMP in 0.1 M sodium phosphate buffer, pH 8.6; and (3) 5.5 μM LH$_2$-AMP in 0.1 M sodium phosphate buffer, pH 8.6.

Relative light yields of reactions of Ppy and PplGR with D-DiMeLH$_2$-AMP. Integration-based light assays conducted as previously described[4] were used to determine relative light yields from D-DiMeLH$_2$-AMP and LH$_2$-AMP with Ppy and PplGR. Integration times were adjusted to insure that at least 95% of the total emitted light was collected for each reaction. In each reaction (0.5 mL), the final concentrations of the reagents were: luciferases (0.32 μM), LH$_2$-AMP (0.12 μM), and D-DiMeLH$_2$-AMP (0.12 μM). Bioluminescence reactions were initiated by rapid injection of the enzymes into 0.1 M sodium phosphate buffer, pH 7.8 with LH$_2$-AMP (5 min integration) and 50 mM Tris, pH 8.6 with D-DiMeLH$_2$-AMP (30 min integration). Total light output was corrected for the spectral response of the Hamamatsu 931B photomultiplier tube and the fluorescence quantum yield of 5,5-dimethyloxyluciferin[9].

Steady-state kinetic constants. Values of K_m and V_{max} for LH$_2$, LH2-AMP and D-DiMeLH$_2$-AMP with Ppy were determined from bioluminescence activity assays in which measurements of maximal light intensities were taken as estimates of initial velocities. Data were collected for reactions in 50 mM Tris buffer, pH 8.6, and were analyzed as described[4,7] earlier. The reversible inhibition of Ppy by D-DiMeLH$_2$ and 5,5-dimethyloxyluciferin was assessed in 50 mM Tris buffer, pH 8.6, using assay conditions and methods reported[10] previously. The K_I value for D-DiMeLH$_2$ with respect to LH$_2$ was obtained by varying the concentration of LH$_2$ from 5.0 to 70 μM in the absence or presence of constant concentrations of D-DiMeLH$_2$ (0.8-7.5 μM) while maintaining a constant, saturating Mg-ATP concentration (2.0 mM). The K_I for 5,5-dimethyloxyluciferin with respect to LH$_2$-AMP was determined by varying the LH$_2$-AMP concentration from 0.15 to 14.0 μM in the absence or presence of constant concentrations of 5,5-dimethyloxyluciferin (2.4 - 24.0 μM).

[9] White, E. H.; Steinmetz, M. G.; Miano, J. D.; Wildes, P. D.; Moreland, R. *J. Am. Chem. Soc.* ***1980***, *102*, 3199-3208.

[10] Branchini, B. R.; Magyar, R. A.; Marcantonio, K. M.; Newberry, K. J.; Stroh, J. G.; Hinz, L. K.; Murtiashaw, M. H. *J. Biol. Chem.* **1997**, *272*, 19359-19364.