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Novel Heterocyclic Analogues of Firefly Luciferin

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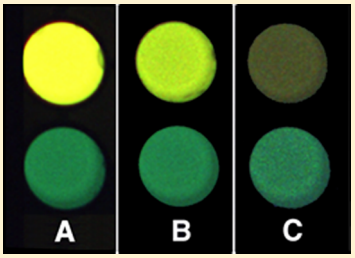
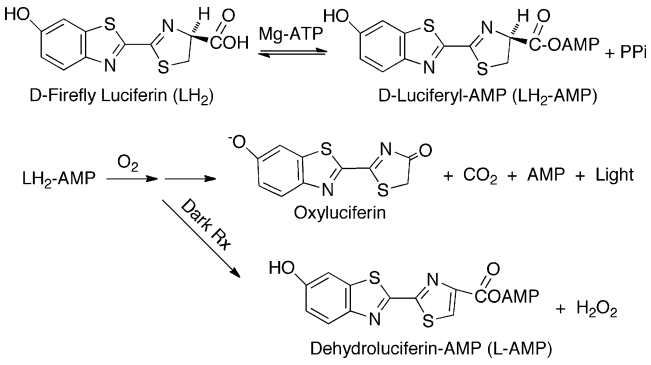
Supporting Information

ABSTRACT: Five novel firefly luciferin analogues in which the benzothiazole ring system of the natural substrate was replaced with benzimidazole, benzofuran, benzothiophene, benzoxazole, and indole were synthesized. The fluorescence, bioluminescence, and kinetic properties of the compounds were evaluated with recombinant *Photinus pyralis* wild type luciferase. With the exception of indole, all of the substrates containing heterocycle substitutions produced readily measurable flashes of light with luciferase. Compared to that of luciferin, the intensities ranged from 0.3 to 4.4% in reactions with varying pH optima and times to reach maximal intensity. The heteroatom changes influenced both the fluorescence and bioluminescence emission spectra, which displayed maxima of 479−528 and 518−574 nm, respectively. While there were some interesting trends in the spectroscopic and bioluminescence properties of this group of structurally similar substrate analogues, the most significant findings were associated with the benzothiophene-containing compound. This synthetic substrate produced slow decay glow kinetics that increased the total light-based specific activity of luciferase more than 4-fold versus the luciferin value. Moreover, over the pH range of 6.2−9.4, the emission maximum is 523 nm, an unusual 37 nm blue shift compared to that of the natural substrate. The extraordinary bioluminescence properties of the benzothiophene luciferin should translate into greater sensitivity for analyte detection in a wide variety of luciferase-based applications.



Beetle bioluminescence is the result of two partial reactions in which the natural substrate firefly (beetle) luciferin (LH_2) is first converted into the corresponding luciferyl-adenylate (LH_2-AMP) by firefly luciferase (Luc) as shown in Scheme 1. In the second half-reaction, excited state oxyluciferin

Scheme 1. Reactions of Firefly Bioluminescence



is formed and subsequently emits a photon of light. A competing oxidative dark reaction leads to the formation of a potent (K_i = 3.8 nM) luciferase inhibitor dehydroluciferin-AMP (L-AMP), a likely cause of the rapid decay of light emission.[1] The bioluminescence process catalyzed by *Photinus pyralis* luciferase is quite efficient, having a bioluminescence quantum yield (Φ_{Bl}) equal to 0.41 ± 0.074.[2] As advanced by White and co-workers[3] and shown in eq 1, the Φ_{Bl} is dependent on the product of three yields: the formation of oxyluciferin (Φ_{Rx}), the formation of oxyluciferin in the excited state (Φ_{ES}), and the fluorescence quantum yield (Φ_{Fl}) of oxyluciferin.

$$\Phi_{Bl} = \Phi_{Rx}\Phi_{Fl}\Phi_{ES} \quad (1)$$

While many luciferin analogues have been reported[4−16] for a variety of applications, the focus of this investigation is on substrates that react directly with luciferase and Mg-ATP to produce light. The synthesis and evaluation of LH_2 analogues began in the laboratory of E. H. White[4,5] shortly after the structure proof and first synthesis of LH_2 was accomplished.[17] The first analogues contained variations in the position and number of hydroxyl groups on the luciferin benzothiazole ring, and later analogues were made containing an additional methyl group[8] and with an amino functionality substituted for the 6′-hydroxyl group.[6] Aminoluciferin proved to be an interesting substrate analogue mainly because it produced considerably bright red-shifted light emission (~605 nm) with Luc. Several recent reports[10−14] have focused on introducing variations into the aminoluciferin structure with quite interesting outcomes. This study is a continuation of a previous one[7,9] that focused on LH_2 analogues in which the benzothiazole moiety was replaced with the naphthalene and quinoline aromatic systems, while maintaining the 2′,6′-substituent pattern. Other than the

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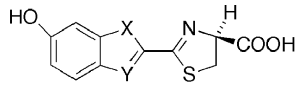
substitution of the benzothiazole system with the coumarin moiety,[13] we are unaware of any additional ring substitutions based on our original strategy (during the preparation of this work, the synthesis of benzimidazolylluciferin was reported[16]). Here we report the preparation and evaluation with recombinant *P. pyralis* luciferase (PpyWT) of five novel LH_2 analogues in which the benzothiazole ring system has been replaced with benzimidazole ($BiLH_2$), benzofuran ($BfLH_2$), benzoxazole ($BoLH_2$), benzothiophene ($BtLH_2$), and indole ($InLH_2$). The properties of the new analogues whose structures are shown in Figure 1 were systematically compared to those of the natural substrate LH_2.



LH_2: X = S, Y = N $BtLH_2$: X = S, Y = CH
$BiLH_2$: X = NH, Y = N $InLH_2$: X = NH, Y = CH
$BoLH_2$: X = O, Y = N $BfLH_2$: X = O, Y = CH

Figure 1. Chemical structures of firefly luciferin (LH_2) and its substrate analogues.

One aim of these studies was to develop new reagents to increase the sensitivity and diversity of enzyme assays based on luciferase bioluminescence measurements. While our results with $BtLH_2$ were quite promising, studies of the unusual properties of this substrate analogue have allowed us to gain a better understanding of the light emission kinetics of the luciferase-catalyzed process.

MATERIALS AND METHODS

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) from Sigma-Aldrich (St. Louis, MO) and firefly luciferin (LH_2) from Promega (Madison, WI). Recombinant firefly luciferase (PpyWT) was prepared as described previously.[18,19] Synthetic procedures and characterization of novel compounds are provided as Supporting Information.

General Methods. Protein concentrations were determined with the Bio-Rad Protein Assay system using bovine serum albumin as the standard. Ultraviolet (UV)−visible spectra were recorded in 25 mM glycylglycine buffer (pH 7.8) with a Perkin-Elmer Lambda 25 spectrometer. Enzyme activity assays were performed with PpyWT, and all data were replicated in triplicate and are reported as means ± the standard deviation.

Luciferase Activity Assays. Bioluminescence activity assays were performed with a custom-built luminometer assembly containing a Hamamatsu R928 PMT and a C6271 HV power supply socket assembly that was described in detail previously.[20] Reactions (0.525 mL final volume) were initiated by the injection of 0.120 mL of 9.0 mM ATP into 8 mm × 50 mm polypropylene tubes containing 0.4 mL of buffer with luciferin or substrate analogues and 5 μL (0.4−4 μg) of enzyme in 20 mM Tris-HCl (pH 7.4 at 4 °C) containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.8 M ammonium sulfate, and 2% glycerol. Assay buffers were 25 mM glycylglycine (pH 8.2) and 50 mM 2-amino-2-methyl-1,3-propanediol (AMPD) (pH 8.5 or 9.1) for $BtLH_2$. The final LH_2 and analogue concentrations were ∼6 times their K_m values. Peak height and integrated intensity values were recorded and corrected for the spectral response of the detector.

Bioluminescence Emission Spectra. Emission spectra were recorded using a Horiba Jobin-Yvon *i*HR imaging spectrometer equipped with a liquid N_2-cooled CCD detector and the excitation source turned off. Data were collected at 25 °C in a 0.8 mL quartz cuvette over the wavelength range of 400−935 nm with the emission slit width set to 10 nm. Reactions (0.525 mL final volume) were initiated by addition of 5 μL of a PpyWT stock solution (15−150 nM final concentration) to cuvettes containing substrate (150 μM) and Mg-ATP (2 mM) in 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) (pH 6.2), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0), 25 mM glycylglycine buffer (pH 7.8 or 8.2), or 50 mM AMPD (pH 8.5, 8.6, 9.1, or 9.4). The pH values were confirmed before and after spectra were obtained. All spectra were corrected for the spectral response of the CCD using a correction curve provided by the manufacturer.

Relative Bioluminescence Quantum Yields. Bioluminescent quantum yields for LH_2 and the substrate analogues were determined from bioluminescence activity assays in which a limiting amount of compound was reacted with an excess of luciferase under single-turnover conditions. Into 0.4 mL of buffer at the optimal pH for each compound (see above) containing 65 nM substrate and 3.25 μM PpyWT was injected 0.12 mL of 9 mM Mg-ATP in the same buffer. The light output was monitored until the initial signal intensity decreased by 99% at a sampling rate of 100 Hz. An additional aliquot of enzyme was added to the spent mixtures, and the emission intensity was monitored to ensure that the reactions were completed. The total integrated light intensities were reported relative to the value obtained with LH_2 at pH 8.2.

Steady State Kinetic Constants. Values of K_m and V_{max} for the substrate analogues were determined as previously described[21] from bioluminescence activity assays in which

Table 1. Fluorescence and Bioluminescence Properties of LH_2 and Its Analogues

substrate	pK_a[a]	pH optimum[b]	fluorescence λ_{max}[c]	fluorescence Φ_{Fl}[d] pH 11.0	fluorescence Φ_{Fl}[d] pH optima	relative bioluminescence Φ_{Bl} (%)
LH_2	8.6	8.2	537	0.83	0.71	100 ± 1.5
$BtLH_2$	9.1	9.1	485	0.33	0.28	70 ± 2.5
$BoLH_2$	8.8	8.5	528	0.71	0.57	0.9 ± 0.1
$BiLH_2$	9.6	8.5	533	0.03	0.02	14 ± 1.0
$BfLH_2$	9.2	8.5	479	0.08	0.07	1.9 ± 0.1
$InLH_2$	10.0	ND[e]	429	0.02	0.01	<0.1

[a] pK_a values are within error of ±0.1. [b] pH optima of reactions with PpyWT values are within error of ±0.1. [c] Fluorescence λ_{max} values are within error of ±1. [d] Fluorescence quantum yields were measured at pH 11.0 and reaction pH optima with 370 nm excitation, and values are within error of ±5%. [e] Data could not be obtained for $InLH_2$ because of low bioluminescence activity.

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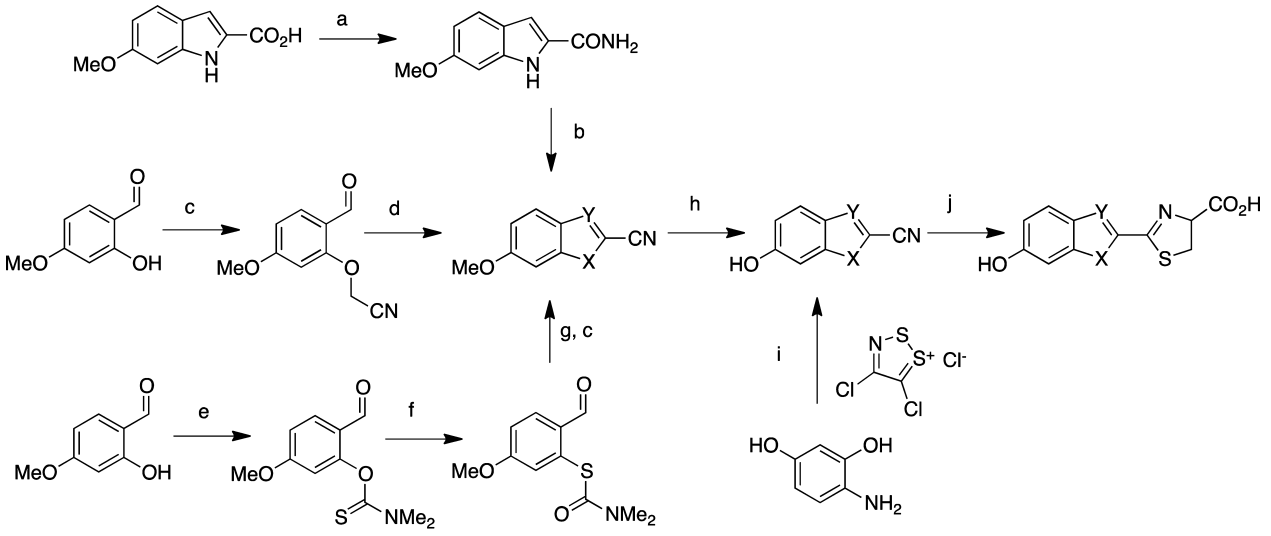
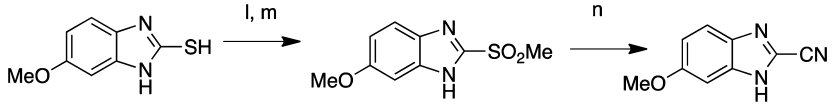
Scheme 2. Synthetic Pathways to Luciferin Analogues



a. i. $SOCl_2$, DMF(cat) ii. NH_4OH, H_2O b. $POCl_3$ c. $BrCH_2CN$, K_2CO_3, THF d. K_2CO_3, DMF, 100 C e. $ClC(S)NMe_2$, DBU, DMF 0 C. f. PhMe, 180 C. g. NaOH, H_2O h. pyridine HCl, 220 C. i. THF/DCM, 0 C, then 150 C 10 min. j. D-cysteine, H_2O/MeCN.



l. MeI, TEA. m. mCPBA. n. KCN, DMSO.

Table 2. Kinetic Properties of LH_2 and Its Analogues[a]

substrate	K_m (μM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	rise time[c] (s)	decay time to 10%[c] (s)	relative specific activity[d]	
						flash height	integration
LH_2	28 ± 2	1.9×10^{-1}	6.78 ± 0.14	0.36	23	100	100
$BtLH_2$	61 ± 2	6.7×10^{-3}	0.11 ± 0.002	6	5700	4.4	421
$BoLH_2$	176 ± 26	1.8×10^{-3}	0.01 ± 0.001	0.35	500	1.0	13
$BiLH_2$	20 ± 5	5.1×10^{-4}	0.03 ± 0.001	0.6	400	0.3	0.4
$BfLH_2$	70 ± 4	6.3×10^{-4}	0.01 ± 0.001	4	150	0.6	1.7

[a]Data could not be obtained for $InLH_2$ because of low bioluminescence activity. [b]k_{cat} values are within error of ±5%. [c]Rise (time to reach maximal intensity) and decay times are within error of ±10%. [d]Relative specific activity values are within error of ±10%.

measurements of maximal light intensities were taken as estimates of initial velocities. Activity assays were performed at the pH optimum for each compound (Table 1). Briefly, the measurements were taken with Mg-ATP at a saturating level (2 mM) and varying concentrations of substrate: LH_2, 2−500 μM; $BtLH_2$, 3−750 μM; $BoLH_2$, 0.6−1.0 mM; $BiLH_2$, 5−200 μM; and $BfLH_2$, 5−600 μM. Data were collected and analyzed using Enzyme Kinetics Pro (Syntex, Palo Alto, CA).

RESULTS AND DISCUSSION

Luciferin Analogue Synthesis and Characterization. The novel luciferin analogues were synthesized as shown in Scheme 2. All were accessed via reaction of D-cysteine with the corresponding nitrile precursor under mild aqueous conditions. While the appropriate precursor for benzoxazole luciferin $BoLH_2$ was prepared directly by a treatment of aminoresorcinol with Appel's salt,[22] the other analogues made use of a phenolic methyl ether protecting group that was removed by a brief treatment with molten pyridinium hydrochloride. Synthesis of

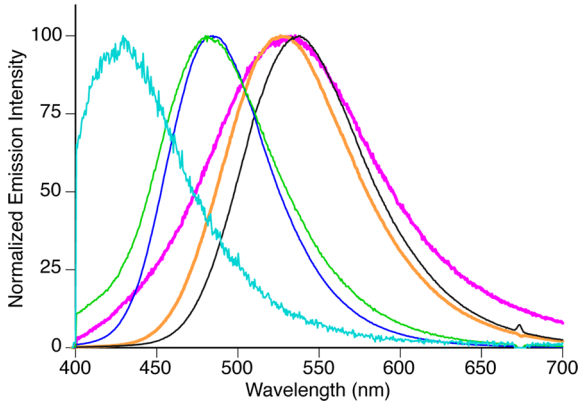


Figure 2. Normalized fluorescence emission spectra of LH_2 (black) and its analogues ($InLH_2$, cyan; $BfLH_2$, green; $BtLH_2$, blue; $BoLH_2$, orange; $BiLH_2$, magenta) recorded at the pH optima for bioluminescence (Table 1). Spectra were acquired with 330 nm excitation as described in detail in the Supporting Information.

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Table 3. Bioluminescence Emission Spectra[a]

substrate	bioluminescence λ_{max} (nm)					
	pH 6.2	pH 7.0	pH 7.8	pH 8.6	pH 9.4	pH optima
LH_2	613 (65)	562 (89)	561 (73)	560 (68)	559 (69)	560 (71)
$BtLH_2$	525 (61)	522 (56)	523 (57)	523 (57)	523 (57)	523 (57)
$BoLH_2$	604 (63)	580 (94)	561 (86)	557 (75)	557 (71)	557 (77)
$BiLH_2$	614 (96)	600 (103)	577 (96)	574 (80)	570 (78)	574 (80)
$BfLH_2$	ND[b]	527 (47)	519 (57)	518 (58)	516 (62)	518 (57)

[a]Bioluminescence λ_{max} values are within error of ±1. Values in parentheses are bandwidths (nanometers) measured at half-maximal intensity. [b]Data could not be obtained because of low bioluminescence activity.

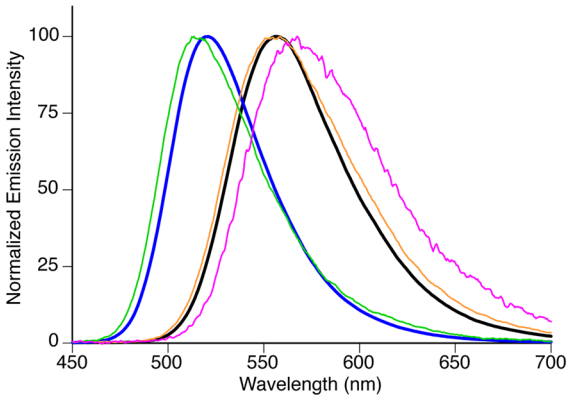

Figure 3. Normalized bioluminescence emission spectra of LH_2 (black) and its analogues ($BfLH_2$, green; $BtLH_2$, blue; $BoLH_2$, orange; $BiLH_2$, magenta) recorded at the pH optima for bioluminescence (Table 1). Additional experimental details are described in Materials and Methods.

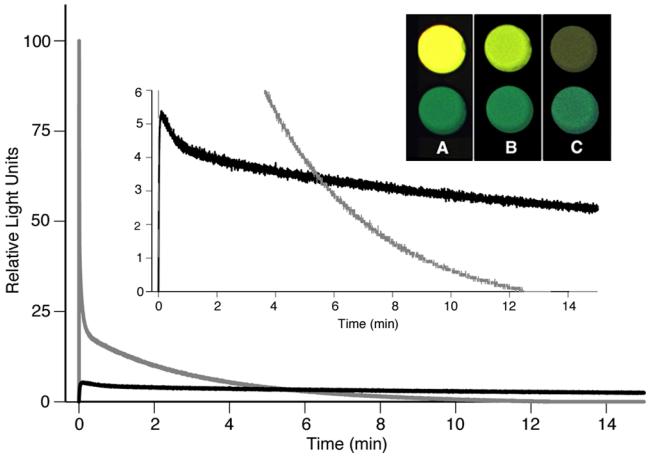

Figure 4. Light emission time courses for PpyWT-catalyzed bioluminescence reactions of LH_2 at pH 8.2 and $BtLH_2$ at pH 9.1 recorded as described in Materials and Methods. The top right inset shows bioluminescence emission images of in vitro reactions of LH_2 (top) and $BtLH_2$ (bottom) taken (A) 5 s, (B) 5 min, and (C) 30 min after the initiation of light reactions. Assays (0.130 mL volume) in 25 mM glycylglycine buffer (pH 8.2) for LH_2 or 50 mM AMPD (pH 9.1) for $BtLH_2$ contained 2.0 mM Mg-ATP, 1 μg of enzyme, and substrate concentrations ~6 times their K_m values. All images were obtained with a ChromaScan Lite Imaging System.

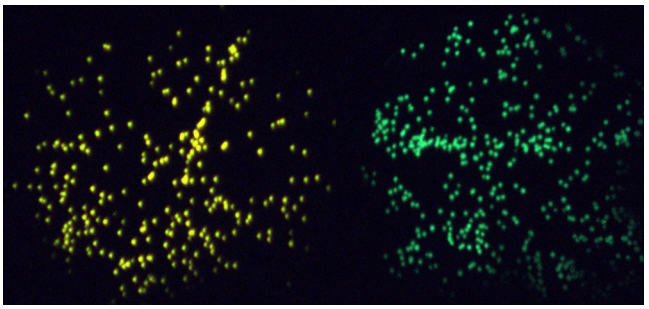

Figure 5. Bioluminescence imaging of *E. coli* colonies on nitrocellulose filters expressing PpyWT. Light reactions were initiated by soaking nitrocellulose filters with 0.6 mL of 1 mM solutions of LH_2 (left) and $BtLH_2$ (right) in 0.1 M sodium citrate buffer (pH 5.5). After ~3 min, images were obtained with a ChromaScan Lite Imaging System.

the indole core has been previously reported and was achieved via a similar route.[23] The nitrile intermediates for the benzofuran and benzothiophene luciferins were approached from a common 4-methoxysalicylaldehyde precursor. In the case of the benzofuran luciferin, direct reaction with a haloacetonitrile and subsequent condensation of the activated methylene with the proximal aldehyde afforded the methoxy benzofuranonitrile. The latter transformation was somewhat sensitive to reaction conditions, as the nitrile was prone to hydration to form the amide; however, potassium carbonate in anhydrous DMF at high temperatures proved to be quite effective.[24] The benzothiophene core was accessed by thiocarbamoylation of the same salicylaldehyde, followed by a heat-induced rearrangement[25] and subsequent hydrolysis to furnish the thiophenol. The conditions used to alkylate the thiophenol intermediate resulted in spontaneous condensation of the aldehyde with the activated methylene to yield the desired benzothiophene nitrile. Finally, the benzimidazole derivative $BiLH_2$ was synthesized from the commercially available 2-thiobenzimidazole via methylation, oxidation to the sulfone, and subsequent displacement with potassium cyanide. The methoxybenzimidazole-2-carbonitrile was elaborated to $BiLH_2$ by standard deprotection and cyclization with D-cysteine. Additionally, chiral HPLC analysis suggested that the substrates were obtained in >99% enantiomeric excess (ee). Racemization occurred very slowly in solid form, such that, for example, a sample of $BoLH_2$ still retained an ee of >95% after being stored for more than 5 years at −80 °C.

As expected,[16] the ^1H and ^{13}C NMR spectra of the $BiLH_2$ analogue displayed peak broadening of the resonances caused by rapid tautomerization of the benzimidazole ring in solution. It was not possible to determine the extent to which $BiLH_2$ exists as the 5′- or 6′-hydroxy-substituted compound at the luciferase active site. While this ambiguity is problematic, especially because the 5′-substituted regioisomer is expected to be inactive in light production, the results and interpretations that follow were made using the assumption that $BiLH_2$ exists solely as the 6′-hydroxy-substituted analogue.

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Kinetic Properties with PpyWT. Prior to determining the performance of the substrate analogues with PpyWT, we first determined the optimal assay pH (Table 1) based on the intensity of the flash height produced with Mg-ATP. We then compared the properties of the compounds at their respective pH optima, which ranged from 8.2 to 9.1, throughout the rest of the study. Little variation in the K_m values was observed with the exception that the value for $BoLH_2$ was unexpectedly 6-fold higher than that of LH_2. The parameters K_m, k_{cat}, and k_{cat}/K_m were determined from assays performed at the respective pH optima (Table 1) at which the peak intensities were taken as measures of initial velocity. For PpyWT, the highest k_{cat} and k_{cat}/K_m values, measures of the catalytic efficiency of PpyWT with the analogues, were obtained with $BtLH_2$ (Table 2). Additionally, the emission kinetics (rise and decay times) varied significantly (Table 2). While LH_2, $BoLH_2$, and $BiLH_2$ with Y = N (Figure 1), reached maximal intensity from 0.35 to 0.6 s, $BtLH_2$ and $BfLH_2$ with Y = CH had rise times of 6 and 4 s, respectively. $BtLH_2$ had the most sustained emission as indicated by a decay time of 95 min, which greatly exceeded the value for LH_2 of 23 s. This "glow" type kinetic profile is unusual for PpyWT, which typically displays strong product inhibition and flash kinetics, and contributed to the high relative bioluminescence quantum yield of $BtLH_2$ as discussed below.

Spectral Emission Properties. The fluorescence emission spectra (Figure 2) of the substrate analogues were recorded using a common excitation wavelength of 330 nm; the fluorescence emission maxima and quantum yields (Φ_{Fl}) are summarized in Table 1. The emission maxima varied from 429 nm ($InLH_2$) to 537 nm (LH_2) and the quantum yields (measured at pH 11.0) from 0.01 ($InLH_2$) to 0.83 (LH_2). Additionally, the fluorescence emission maxima of the analogues varied only slightly (≤10 nm) over the pH range of 7.8−11.0. With respect to emission maxima at optimal pH, the substrates fell into two groups: (1) LH_2, $BoLH_2$, and $BiLH_2$ that have λ_{max} values of ∼530 nm (range of 528−537 nm) and (2) $InLH_2$, $BfLH_2$, and $BtLH_2$ that have λ_{max} values in the range of 429−485 nm. The clear distinguishing factor is the absence or presence of a nitrogen atom (Y = N in Figure 1) that is associated with longer wavelength emission.

The range of bioluminescence emission maxima (Table 3 and Figure 3) at the respective pH optima for the substrate−luciferase reactions varied 56 nm from 518 nm ($BfLH_2$) to 574 nm ($BiLH_2$). When Y = CH, green emission (518−523 nm) is observed for $BtLH_2$ and $BfLH_2$. Yellow-green light (556−574 nm) was observed with LH_2, $BiLH_2$, and $BoLH_2$ that have Y = N. Data could not be obtained for $InLH_2$ because of extremely low bioluminescence activity, and this analogue was not further evaluated.

The spectral shift of approximately 50 nm over the pH range of 6.2−9.4 with LH_2 is an example of the pH sensitivity of the true firefly luciferases (Table 3). Only $BoLH_2$ and $BiLH_2$ exhibit pH behavior very similar to that of LH_2, while $BtLH_2$ and $BfLH_2$ are almost insensitive to pH change. Apparently, fluorescence and bioluminescence emission as well as pH sensitivity and reaction rise times are related to whether Y = CH or Y = N. In the crystal structures of *Lunularia cruciata* and *P. pyralis* luciferases[26,27] in complex with an inhibitor structurally similar to L-AMP, the N atom at the Y position of the inhibitor is hydrogen bonded to S347 through an intervening water molecule at the active site. A disruption of this interaction may be responsible for the resistance to red-shifting emission at low pH, the long wavelength bioluminescence, and slow rise times.

Relative Bioluminescence Quantum Yields (Φ_{Bl}). The Φ_{Bl} values (relative to the value obtained with LH_2) were measured with excess enzyme under single-turnover conditions (Table 1). The results also fall into two groups: (1) those with high (≥70%) values (LH_2 and $BtLH_2$) and (2) those with very low (≤14%) values ($BiLH_2$, $BfLH_2$, $BoLH_2$, and $InLH_2$). Here there is a correlation with the electronegativity of the X position atom (Figure 1). Higher values are associated with the low electronegativity of S, while O and N produce lower Φ_{Bl} values. This effect is especially powerful as one might expect that the Φ_{Bl} values would follow the fluorescence quantum yield (Φ_{Fl}) trend (Table 1), suggesting, for example, that $BoLH_2$ (Φ_{Fl} = 0.71) should have the highest Φ_{Bl} value among the analogues. Instead, $BoLH_2$ produced little light (∼1% compared to LH_2). Evidently, the high electronegativity of the oxygen atom interferes with the efficient formation of the excited state analogue oxyluciferins (Φ_{ES}) and/or causes the corresponding adenylates to form dehydroluciferin-like product in great excess over oxyluciferin (Φ_{Rx}). Possibly too, the level of adenylate formation is severely reduced (Scheme 1). In marked contrast, $BtLH_2$ efficiently produces light with a relative Φ_{Bl} equal to 70% of that of LH_2 despite having a Φ_{Fl} of 0.33, only 40% of the LH_2 value. This analogue may be capable of producing a greater percentage of analogue oxyluciferin in the excited state (Φ_{ES}) and/or with a higher analogue oxyluciferin:L-AMP ratio (Φ_{Rx}). The latter is likely occurring as evidenced by the extended decay when substrate is in excess. The slow decay may be due to slower PpyWT inhibition because less analogue L-AMP is formed and/or because it has a higher K_i value than L-AMP. A lower level of product inhibition by the oxyluciferin analogue may also contribute. These arguments, like those above, assume that the heterocyclic analogues bind in the same relative orientation as LH_2 with respect to rotation about the C2−C2′ bond. Otherwise, the relative position of the 6′-hydroxyl at the active site will be altered and would likely diminish light emission.

$BtLH_2$ Is a Promising Luc Substrate. Firefly luciferase assays based on ATP detection, reporter gene detection, and in vivo bioluminescence imaging can be performed more conveniently and with greater sensitivity when the signal decays slowly displaying glow rather than "flash" kinetics. Interestingly, although the initial intensity of $BtLH_2$ is only ∼4% of that produced with LH_2, this intensity decays so slowly (Figure 4) that over 1.5 h, ∼4-fold greater total light is emitted than with the natural substrate (Table 2). To demonstrate the importance of the sustained light emission of $BtLH_2$, we recorded images for reaction mixtures with identical amounts of PpyWT and Mg-ATP and saturating concentrations of substrates under optimal conditions, pH 8.2 for LH_2 and pH 9.1 for the analogue. Initially, the LH_2-containing reaction mixture is brighter, but after 5 min, the reaction mixtures are of approximately equal intensity (Figure 4, inset). However, the bright emission with $BtLH_2$, but not LH_2, persisted even after 30 min (Figure 4, inset). Also, the blue-shifted emission of PpyWT with $BtLH_2$ will provide better signal separation with red bioluminescence signals than is possible with LH_2. Additionally, we evaluated the potential of $BtLH_2$ for imaging studies by using it to visualize single *Escherichia coli* colonies expressing PpyWT (Figure 5). Surprisingly, despite the higher pH optimum of bioluminescence with $BtLH_2$, the signal

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intensities produced in bacteria with the analogue and LH_2 are very similar.

We have developed synthetic methods for the preparation of five novel LH_2 analogues. Among them, $BtLH_2$ displays the most promise as an alternative substrate for Luc in applications where it is advantageous to have emission that is blue-shifted and longer-lived than that typically observed with the natural substrate. These attributes have been achieved without the necessity of chemical additives. Studies are in progress to develop firefly luciferases that selectively enhance the properties of $BtLH_2$ compared to those of the natural substrate.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures; procedures for determining substrate pK_a values, bioluminescence reaction pH optima, and fluorescence spectra; and the dependence of bioluminescence activity on pH. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

$BfLH_2$, D-benzofuranylluciferin; $BiLH_2$, D-benzimidazolylluciferin; $BoLH_2$, D-benzoxazolylluciferin; $BtLH_2$, D-benzothiophenylluciferin; $InLH_2$, D-indolylluciferin; L-AMP, dehydroluciferyladenylate; LH_2, D-firefly luciferin; Luc, *P. pyralis* luciferase (EC 1.13.12.7); PpyWT, recombinant *P. pyralis* luciferase containing the additional N-terminal GlyProLeuGlySer peptide.

■ REFERENCES

(1) Ribeiro, C., and da Silva, J. (2008) Kinetics of inhibition of firefly luciferase by oxyluciferin and dehydroluciferyl-adenylate. *Photochem. Photobiol. Sci. 7*, 1085−1090.

(2) Ando, Y., Niwa, K., Yamada, N., Enomot, T., Irie, T., Kubota, H., Ohmiya, Y., and Akiyama, H. (2008) Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission. *Nat. Photonics 2*, 44−47.

(3) White, E. H., Rapaport, E., Seliger, H. H., and Hopkins, T. A. (1971) Chemi- and bioluminescence of firefly luciferin. Efficient chemical production of electronically excited states. *Bioorg. Chem. 1*, 92−122.

(4) White, E. H., Worther, H., Field, G. F., and McElroy, W. D. (1965) Analogs of Firefly Luciferin. *J. Org. Chem. 30*, 2344−2348.

(5) White, E. H., and Worther, H. (1966) Analogs of firefly luciferin. 3. *J. Org. Chem. 31*, 1484−1488.

(6) White, E. H., Worther, H., Seliger, H. H., and McElroy, W. D. (1966) Amino analogs of firefly luciferin and biological activity thereof. *J. Am. Chem. Soc. 88*, 2015−2019.

(7) Branchini, B. R., Hayward, M. M., Bamford, S., Brennan, P. M., and Lajiness, E. J. (1989) Naphthylluciferin and Quinolylluciferin: Green and Red-Light Emitting Firefly Luciferin Analogs. *Photochem. Photobiol. 49*, 689−695.

(8) Farace, C., Blanchot, B., Champiat, D., Couble, P., Declercq, G., and Millet, J. L. (1990) Synthesis and characterization of a new substrate of *Photinus pyralis* luciferase: 4-Methyl-D-luciferin. *J. Clin. Chem. Clin. Biochem. 28*, 471−474.

(9) Branchini, B. R. (2000) Chemical synthesis of firefly luciferin analogs and inhibitors. *Methods Enzymol. 305*, 188−195.

(10) Shinde, R., Perkins, J., and Contag, C. H. (2006) Luciferin derivatives for enhanced in vitro and in vivo bioluminescence assays. *Biochemistry 45*, 11103−11112.

(11) Woodroofe, C. C., Shultz, J. W., Wood, M. G., Osterman, J., Cali, J. J., Daily, W. J., Meisenheimer, P. L., and Klaubert, D. H. (2008) N-alkylated 6′-aminoluciferins are bioluminescent substrates for Ultra-Glo and QuantiLum luciferase: New potential scaffolds for bioluminescent assays. *Biochemistry 47*, 10383−10393.

(12) Reddy, G. R., Thompson, W. C., and Miller, S. C. (2010) Robust Light Emission from Cyclic Alkylaminoluciferin Substrates for Firefly Luciferase. *J. Am. Chem. Soc. 132*, 13586−13587.

(13) Takakura, H., Sasakura, K., Ueno, T., Urano, Y., Terai, T., Hanaoka, K., Tsuboi, T., and Nagano, T. (2010) Development of Luciferin Analogues Bearing an Amino Group and Their Application as BRET Donors. *Chem. Asian J. 5*, 2053−2061.

(14) Takakura, H., Kojima, R., Urano, Y., Terai, T., Hanaoka, K., and Nagano, T. (2011) Aminoluciferins as Functional Bioluminogenic Substrates of Firefly Luciferase. *Chem. Asian J. 6*, 1800−1810.

(15) Conley, N. R., Dragulescu-Andrasi, A., Rao, J. H., and Moerner, W. E. (2012) A Selenium Analogue of Firefly D-Luciferin with Red-Shifted Bioluminescence Emission. *Angew. Chem., Int. Ed. 51*, 3350−3353.

(16) McCutcheon, D. C., Paley, M. A., Steinhardt, R. C., and Prescher, J. A. (2012) Expedient Synthesis of Electronically Modified Luciferins for Bioluminescence Imaging. *J. Am. Chem. Soc. 134*, 7604−7607.

(17) White, E. H., McCapra, F., and Field, G. F. (1963) The structure and synthesis of firefly luciferin. *J. Am. Chem. Soc. 85*, 337−343.

(18) Branchini, B. R., Ablamsky, D. M., Murtiashaw, M. H., Uzasci, L., Fraga, H., and Southworth, T. L. (2007) Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem. 361*, 253−262.

(19) Branchini, B. R., Ablamsky, D. M., Davis, A. L., Southworth, T. L., Butler, B., Fan, F., Jathoul, A. P., and Pule, M. A. (2010) Red-emitting luciferases for bioluminescence reporter and imaging applications. *Anal. Biochem. 396*, 290−297.

(20) Branchini, B. R., Ablamsky, D. M., Rosenman, J. M., Uzasci, L., Southworth, T. L., and Zimmer, M. (2007) Synergistic mutations produce blue-shifted bioluminescence in firefly luciferase. *Biochemistry 46*, 13847−13855.

(21) Branchini, B. R., Southworth, T. L., Murtiashaw, M. H., Wilkinson, S. R., Khattak, N. F., Rosenberg, J. C., and Zimmer, M. (2005) Mutagenesis evidence that the partial reactions of firefly bioluminescence are catalyzed by different conformations of the luciferase C-terminal domain. *Biochemistry 44*, 1385−1393.

(22) Cuadro, A. M., and Alvarezbuilla, J. (1994) 4,5-Dichloro-1,2,3-Dithiazolium Chloride (Appels Salt): Reactions with N-Nucleophiles. *Tetrahedron 50*, 10037−10046.

(23) Borza, I., Bozo, E., Barta-Szalai, G., Kiss, C., Tarkanyi, G., Demeter, A., Gati, T., Hada, V., Kolok, S., Gere, A., Fodor, L., Nagy, J., Galgoczy, K., Magdo, I., Agai, B., Fetter, J., Bertha, F., Keseru, G. M., Horvath, C., Farkas, S., Greiner, I., and Domany, G. (2007) Selective

NR1/2B N-Methyl-D-aspartate receptor antagonists among indole-2-carboxamides and benzimidazole-2-carboxamides. *J. Med. Chem. 50*, 901−914.
(24) Hirota, T., Fujita, H., Sasaki, K., Namba, T., and Hayakawa, S. (1986) A Novel Synthesis of Benzofuran and Related-Compounds. 1. The Vilsmeier Reaction of Phenoxyacetonitriles. *J. Heterocycl. Chem. 23*, 1347−1351.
(25) Kolmakov, K. A., and Kresge, A. J. (2008) Synthesis of possible o-thioquinone methide precursors. *Can. J. Chem. 86*, 119−123.
(26) Nakatsu, T., Ichiyama, S., Hiratake, J., Saldanha, A., Kobashi, N., Sakata, K., and Kato, H. (2006) Structural basis for the spectral difference in luciferase bioluminescence. *Nature 440*, 372−376.
(27) Sundlov, J. A., Fontaine, D. M., Southworth, T. L., Branchini, B. R., and Gulick, A. M. (2012) Crystal Structure of Firefly Luciferase in a Second Catalytic Conformation Supports a Domain Alternation Mechanism. *Biochemistry 51*, 6493−6495.