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Novel Heterocyclic Analogs of Firefly Luciferin

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ABREVIATIONS

$BfLH_2$, 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, *Photinus pyralis* luciferase (E.C. 1.13.12.7); PpyWT, recombinant *Photinus pyralis* luciferase containing the additional N-terminal peptide GlyProLeuGlySer–.

TEXTUAL FOOTNOTES

[§]During the preparation of this manuscript, the synthesis of benzimidazolylluciferin was reported[16].

ABSTRACT

Five novel firefly luciferin analogs were synthesized in which the benzothiazole ring system of the natural substrate was replaced with: benzimidazole, benzofuran, benzothiophene, benzoxazole and indole. 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 luciferin, the intensities ranged from 0.3% to 4.4% in reactions with varying pH optima and times to reach maximum intensity. The heteroatom changes influenced both the fluorescence and bioluminescence emission spectra, which displayed maxima of 479 nm to 528 nm and 518 nm to 574 nm, respectively. While there were some interesting trends in the spectroscopic and bioluminescence properties of this group of structurally similar substrate analogs, 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 over the luciferin value. Moreover, over the pH range 6.2 to 9.4 the emission maximum is 523 nm, an unusual 37 nm blue-shift compared to 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.

INTRODUCTION

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 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 coworkers[3] and shown in Equation 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.

$$\Phi_{Bl} = \Phi_{Rx} \cdot \Phi_{Fl} \cdot \Phi_{ES} \qquad \text{(Equation 1)}$$

While many luciferin analogs 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 analogs 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 analogs contained variations in the position and number of hydroxyl groups on the luciferin benzothiazole ring, and later analogs 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 analog mainly because it produced considerably bright red-shifted light

emission (~605 nm) with Luc. Several recent reports[10-14] have focused on introducing variations to the aminoluciferin structure with quite interesting outcomes. This study is a continuation of a previous one[7, 9] that focused on LH_2 analogs in which the benzothiazole moiety was replaced with the naphthalene and quinoline aromatic systems, while maintaining the 2′-, 6′- substituent pattern. Other than the substitution of the benzothiazole system with the coumarin moiety[13], we are unaware of any additional ring substitutions based on our original strategy[§]. Here we report the preparation and evaluation with recombinant *P. pyralis* luciferase (PpyWT) of five novel LH_2 analogs 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 analogs whose structures are shown in Figure 1 were systematically compared to those of the natural substrate LH_2.

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 on the unusual properties of this substrate analog have enabled 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 in the 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 the mean ± 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 x 50-mm polypropylene tubes containing 0.4 mL of buffer with luciferin or substrate analogs 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 pH 9.1 for $BtLH_2$. The final LH_2 and analog 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 obtained 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 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 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-piperazineethane-sulfonic acid (HEPES), pH 7.0; 25 mM glycylglycine buffer, pH 7.8 or 8.2; 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 analogs 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, 0.12 mL of 9 mM Mg-ATP in the same buffer was injected. 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 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 analogs were determined as previously described[21] from bioluminescence activity assays in which 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 made 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 software (Syntex, Palo Alto, CA).

RESULTS AND DISCUSSION

Luciferin analog synthesis and characterization. The novel luciferin analogs 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 treating aminoresorcinol with Appel's salt[22], the other analogs made use of a phenolic methyl ether protecting group which was removed by brief treatment with molten pyridinium hydrochloride. Synthesis of 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 temperature proved 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 more than 5 years of storage at -80 °C.

As expected[16], the H1- and C13-NMR spectra of the $BiLH_2$ analog displayed peak broadening of the resonances due to rapid tautomerization of the benzimidazole ring in solution. It was not possible to determine to what extent $BiLH_2$ 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 analog.

Kinetic Properties with PpyWT. Prior to determining the performance of the substrate analogs with PpyWT, we first determined the optimum 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) in 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 analogs, 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 maximum intensity

from 0.35 to 0.6 s, $BtLH_2$ and $BfLH_2$ with Y = CH had rise times of 6 s and 4 s, respectively. $BtLH_2$ had the most sustained emission as indicated by a decay time of 95 min greatly exceeding the 23 s value of LH_2. 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 analogs 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 analogs varied only slightly (≤ 10 nm) over the pH range 7.8 – 11.0. With respect to emission maxima at optimum pH, the substrates fell into two groups: (1) LH_2, $BoLH_2$, and $BiLH_2$ that have λ_{max} = ~530 nm (range 528 nm-537 nm) and (2) $InLH_2$, $BfLH_2$ and $BtLH_2$ that have λ_{max} values in the range 429 nm to 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 nm – 523 nm) is observed for $BtLH_2$, and $BfLH_2$. Yellow-green light (556 nm – 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 bioluminescent activity, and this analog was not further evaluated.

The spectral shift of approximately 50 nm over the pH range 6.2 to 9.4 with LH_2 is an example of the pH sensitivity of the true firefly luciferases (Table 3). Only $BoLH_2$ and $BiLH_2$ show very similar pH behavior to 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 *L. 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, $BtLH_2$) 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 analogs. 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 analog oxyluciferins (Φ_{ES}) and/or causes the corresponding adenylates to form dehydroluciferin-like product in great excess over oxyluciferin (Φ_{Rx}). Possibly too, adenylate formation is severely reduced (Scheme 1). In marked contrast,

$BtLH_2$ efficiently produces light with a relative Φ_{Bl} equal to 70% that of LH_2 despite having a Φ_{Fl} = 0.33, only 40% of the LH_2 value. This analog may be capable of producing a greater percentage of analog oxyluciferin in the excited state (Φ_{ES}) and/or with a higher analog 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 analog L-AMP is formed and/or because it has a higher K_i value than L-AMP. Lower product inhibition by the oxyluciferin analog may also contribute. These arguments, like those above, assume that the heterocyclic analogs 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 hours, ~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 of reactions 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 analog. Initially the LH_2-containing reaction is brighter, but after 5 min the reactions 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 *E. coli* colonies expressing PpyWT (Figure 5). Surprisingly, despite the higher pH optimum of bioluminescence with $BtLH_2$, the signal intensities produced in bacteria with the analog and LH_2 are very similar.

We have developed synthetic methods for the preparation of five novel LH_2 analogs. Among them, $BtLH_2$ displays the most promise as an alternative substrate for Luc in application where it is advantageous to have blue-shifted and longer lived emission than is 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 the natural substrate.

SUPPORTING INFORMATION

Synthetic procedures; procedures for determining substrate pKa values, bioluminescence reaction pH optima, and fluorescence spectra; and figure of 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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Table 1. Fluorescence and bioluminescence properties of LH_2 and analogs

substrate	pK_a[a]	pH optima[b]	fluorescence λ_{max}[c]	fluorescence Φ_{Fl}[d] pH 11.0	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 bioluminescent activity.

Table 2. Kinetic properties of LH_2 and analogs[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	relative specific activity[d] integration
LH_2	28 ± 2	1.9E-01	6.78 ± 0.14	0.36	23	100	100
$BtLH_2$	61 ± 2	6.7E-03	0.11 ± 0.002	6	5700	4.4	421
$BoLH_2$	176 ± 26	1.8E-03	0.01 ± 0.001	0.35	500	1.0	13
$BiLH_2$	20 ± 5	5.1E-04	0.03 ± 0.001	0.6	400	0.3	0.4
$BfLH_2$	70 ± 4	6.3E-04	0.01 ± 0.001	4	150	0.6	1.7

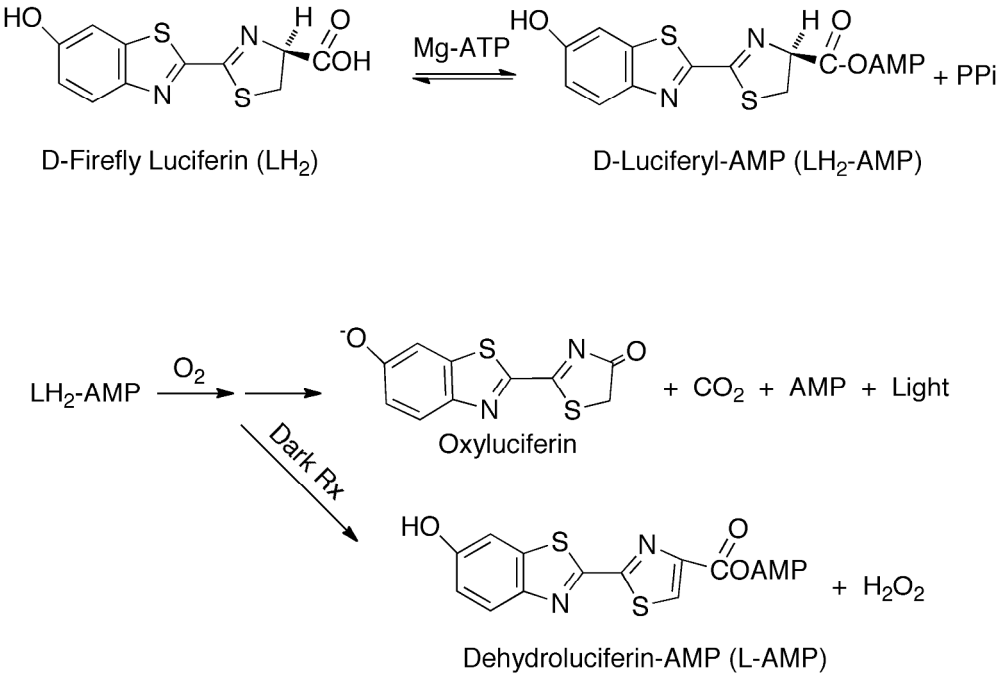
[a]Data could not be obtained for $InLH_2$ because of low bioluminescent activity. [b]k_{cat} values are within error of ± 5%. [c]Rise (time to reach maximum intensity) and decay times are within error of ± 10%. [d]Relative specific activity values are within error of ± 10%.

Table 3. Bioluminescence emission spectra[a]

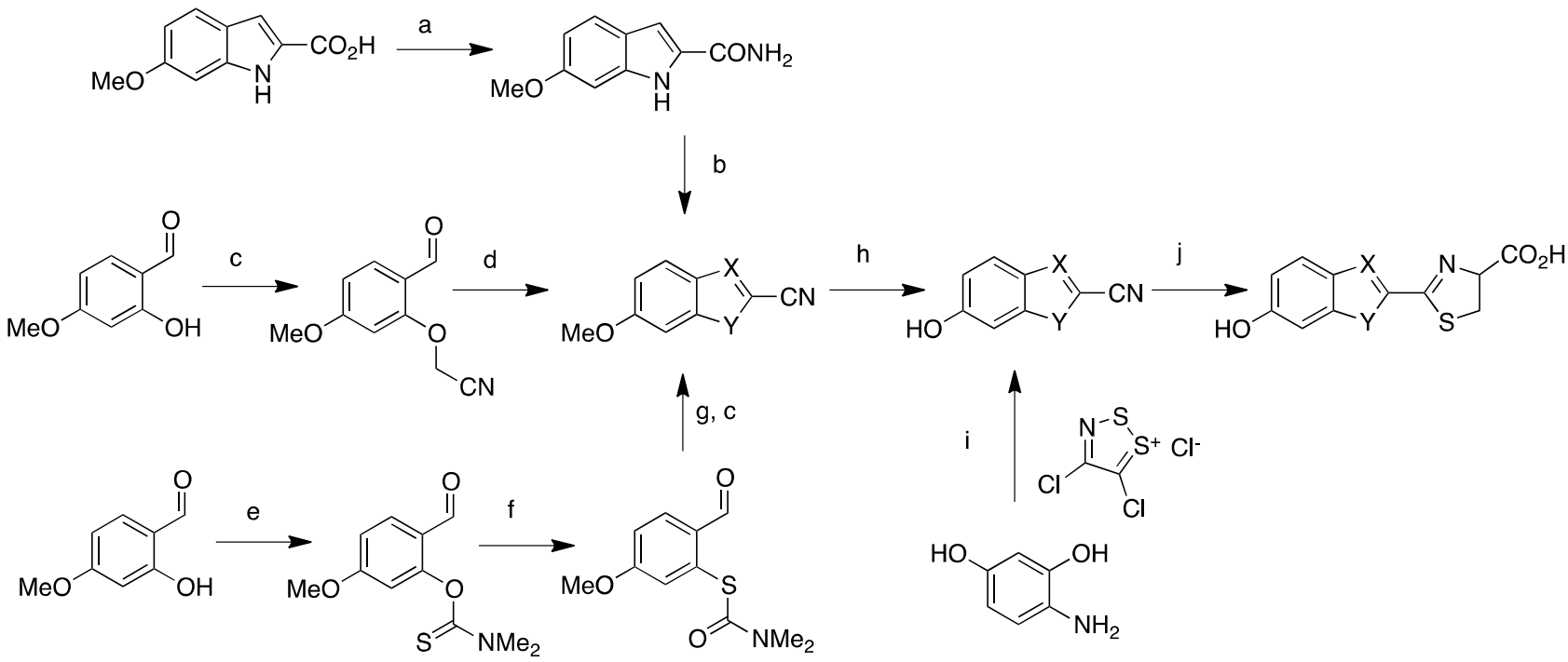
substrate	bioluminescence λ_{max} (nm) pH 6.2	pH 7.0	pH 7.8	pH 8.6	pH 9.4	pH optima
LH_2	613 (65)	562 (89)	561 (73)	560 (68)	559 (69)	560 (71)
$BtLH_2$	525 (61)	522 (56)	523 (57)	523 (57)	523 (57)	523 (57)
$BoLH_2$	604 (63)	580 (94)	561 (86)	557 (75)	557 (71)	557 (77)
$BiLH_2$	614 (96)	600(103)	577 (96)	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 (nm) measured at half-maximum intensity. [b]Data could not be obtained because of low bioluminescent activity.

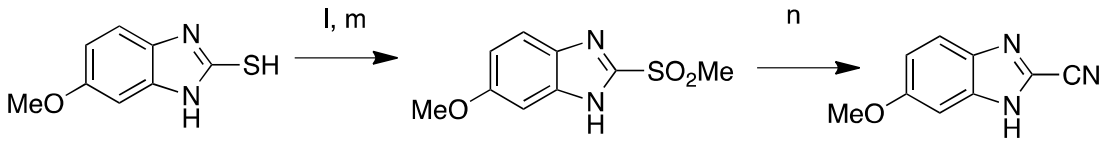
Scheme 1. The reactions of firefly bioluminescence



Scheme 2. Synthetic Pathways to Luciferin Analogs



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.

FIGURE LEGENDS

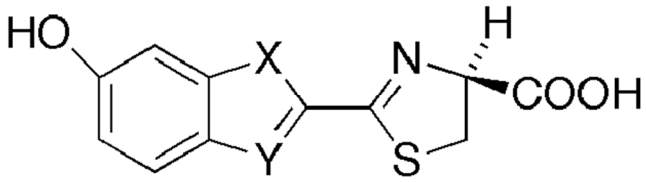
Figure 1. Chemical structures of firefly luciferin (LH_2) and substrate analogs.

Figure 2. Normalized fluorescence emission spectra of LH_2 (black) and analogs ($InLH_2$, 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.

Figure 3. Normalized bioluminescence emission spectra of LH_2 (black) and the analogs ($BfLH_2$, 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 the 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. Top right inset: 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 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.



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

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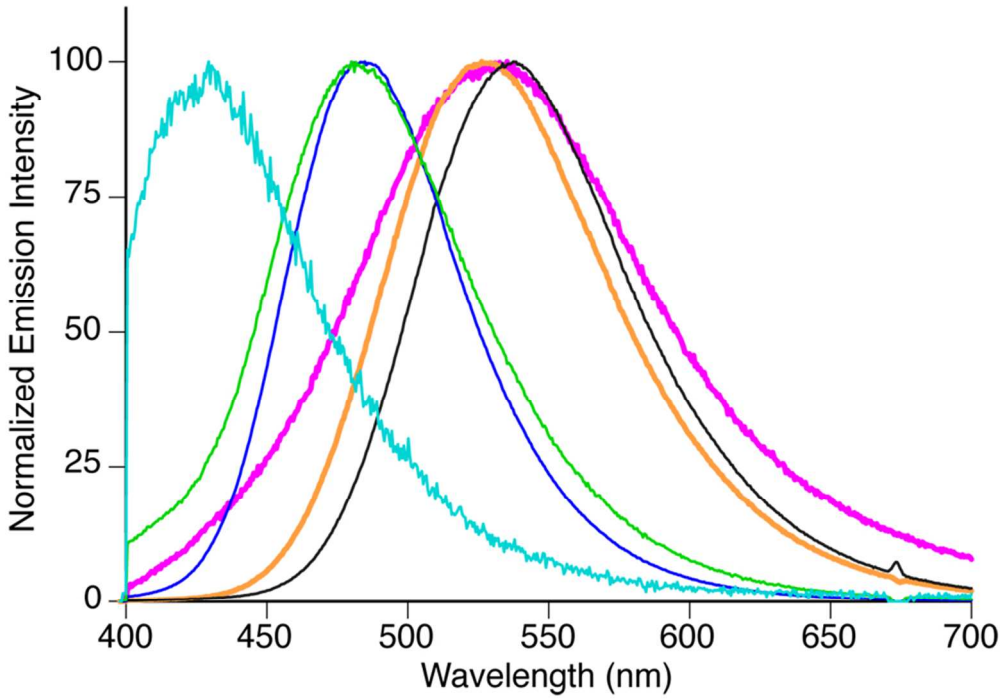

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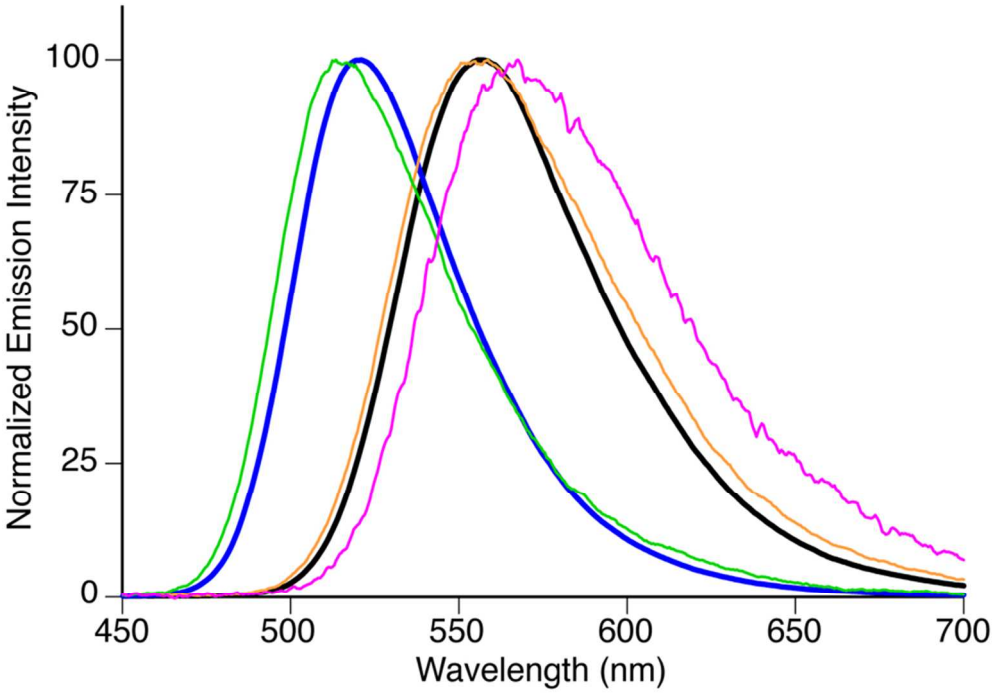

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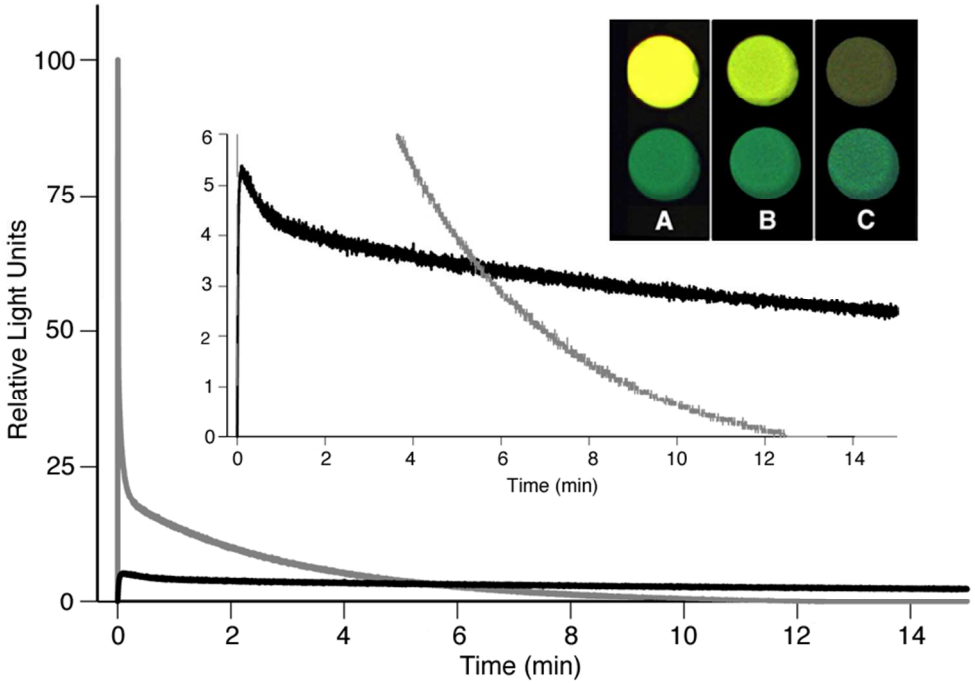

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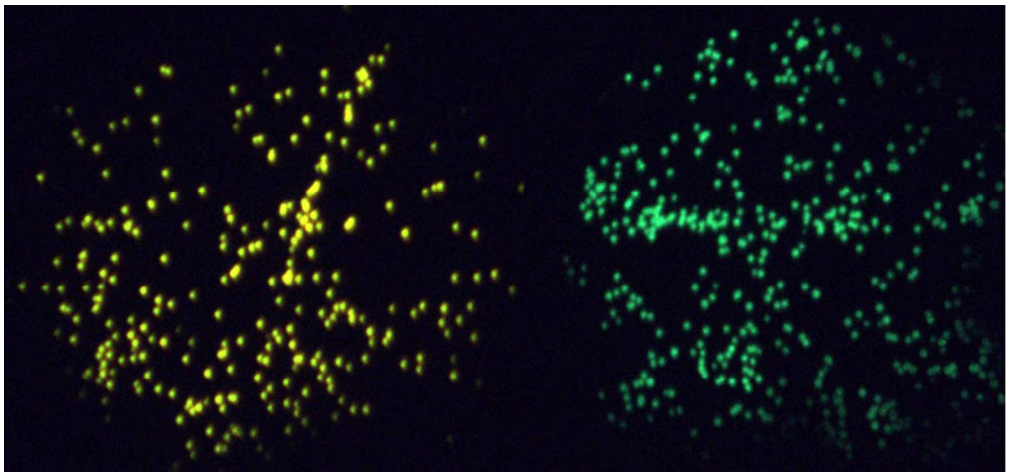

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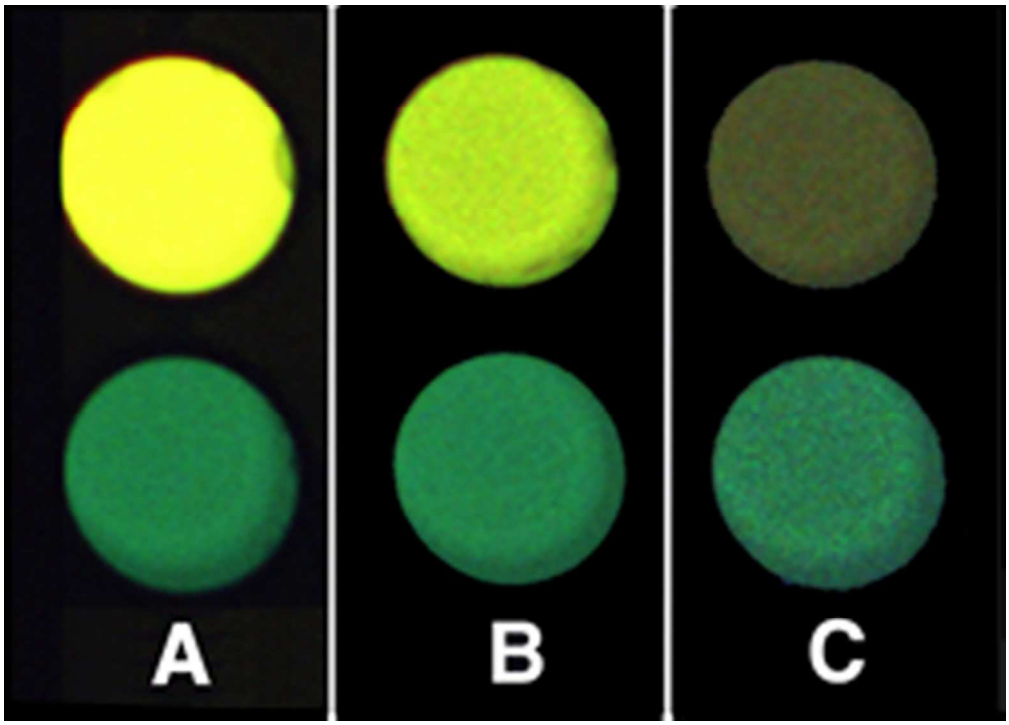

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