

Synthesis and Bioluminescence of 'V'-Shaped Firefly Luciferin Analogues Based on A Novel Benzobisthiazole Core

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The design of π -extended conjugation 'V'-shaped red shifted bioluminescent D-luciferin analogues based on a novel benzobisthiazole core is described. The divergent synthetic route allowed access to a range of amine donor substituents through an S_NAr reaction. In spectroscopic studies, the 'V'-shaped luciferins exhibited narrower optical band gaps, more red-shifted absorption and emission spectra than D-luciferin. Their bioluminescence characteristics were recorded against four different luciferases (PpyLuc, FlucRed, CBR2 and PLR3). With

native luciferase PpyLuc, the 'V'-shaped luciferins demonstrated more red-shifted emissions than D-luciferin (λ_{bi} = 561 nm) by 60 to 80 nm. In addition, the benzobisthiazole luciferins showed a wide range of bioluminescence spectra from the visible light region (λ_{bi} = 500 nm) to the nIR window (> 650 nm). The computational results validate the design concept which can be used as a guide for further novel D-luciferin analogues based upon other 'V'-shaped heterocyclic cores.

Introduction

Bioluminescence (BL) is the generation of light by living organisms. In most cases this involves the catalytic oxidation of a small molecule, luciferin, by an enzyme, luciferase. One of the most popular luciferins is firefly luciferin (D-luciferin (1), LH_2), its structure consisting of a 6-hydroxylbenzothiazole linked to a chiral carboxyl thiazoline ring derived from D-cysteine (Figure 1).^[1] Firefly bioluminescence has been developed for non-invasive bioluminescence imaging (BLI) to monitor, for example, biological process *in-vivo* such as cancer metastasis^[2] or the detection of reactive oxygen species.^[3] The emission wavelength of firefly luciferin can vary between 530–630 nm^[4] dependent upon the microenvironment in the luciferase, pH and temperature. Light in this wavelength range is absorbed by tissue, melanin and hemoglobin that results in reduced

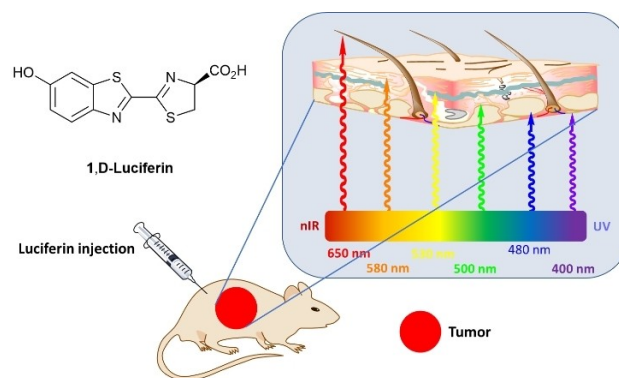


Figure 1. The structure of D-Luciferin and transmission of various photon wavelengths through mammalian skin.

penetration and decreased resolution of the emitted light. Therefore, the development of bioluminescent probes with emission wavelengths much above 600 nm, where light is more transmissible through blood and tissue, above that possible with luciferase mutation alone, is desirable to achieve better penetration and resolution in the BLI of animal systems (Figure 1).

Many successful attempts at tuning the bioluminescence emission wavelength of D-luciferin derivatives have been reported since the first investigations by White.^[5] These synthetic luciferins have been shown to emit red to nIR photons (from 600 nm to ~760 nm) and in some cases have been verified to give better penetration through animal tissue in comparison to native D-luciferin. White, a pioneer in luciferin research, synthesized amino luciferin 2 based on the D-luciferin skeleton.^[6] The replacement of the 6-hydroxyl group of D-luciferin to a primary amine group gave red-shifted bioluminescence emission at 593 nm with firefly luciferase (Fluc)

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(Figure 2) compared to D-luciferin which has maximum emission at 562 nm. This was rationalized by the amine donor making the system more electron rich than the parent oxygen donor of D-luciferin. Inspired by White's work, a series of amino luciferin analogues with push-pull structures were later developed to manipulate the emission wavelengths above 600 nm. The substitution of the sulfur atom to a selenium provided amino selenium luciferin **3**. This simple modification allowed the emission wavelength to be shifted to 600 nm from 593 nm. However, compared to its parent analogue **2**, the number of photons emitted by **3** was 26% lower due to the heavy atom effect.^[7] In contemporaneous studies, Miller investigated a series of ring-fused cyclic amino luciferins, working on the premise that hindrance to rotation around the benzothiazole-N donor bond would increase conjugation of the N lone pair, resulting in better extension of the emission wavelength.^[8] Two of Miller's most efficient analogues CycLuc8 **4** and CycLuc10 **5** share the same benzothiazole core structure with a cyclic fused tertiary amine as the electron donating group (EDG), differing only in the point of ring fusion. The 'V'-shaped **5** structure exhibited a longer red-shifted fluorescence emission than the linear form **4**. This trend was reproduced with the bioluminescence emission of **5** at 642 nm being 32 nm more red shifted than **4** with wild type luciferase.^[8b] Interestingly time-dependent density functional theory (TD-DFT) calculations predicted the red shifted fluorescence emission of **5** and suggested that luciferins modified at the C7' position would be red shifted compared to their C5'-modified isomers.^[9] Elongation of conjugation by introducing alkenyl group(s) between the two aromatic moieties is one of the most successful methods to reach near infrared (nIR) wavelength. The seminal example of this was demonstrated by Iwano *et al.* with the synthesis of Luciferin **6**, and other analogues, that exhibited bioluminescence emission at 675 nm with wild type *P. pyralis* luciferase (PpyWT).^[10] Taking inspiration from Iwano's work, we reported the first small molecule luciferin (**7**, iLH₂) with nIR emission > 700 nm; at 706 nm^[11] with mutant luciferase x5 S284T, 730 nm with

luciferase CBR2^[12] and without resorting to bioluminescence resonance energy transfer (BRET). This nIR luciferin **7**, despite showing the usual weaker light output and lower bio-activity than D-luciferin, could be used for nIR dual bioluminescence imaging to monitor both tumour burden and CAR T cell therapy within a systemically induced mouse tumour model.^[13] In 2018, Kiyama *et al.* constructed diverse luciferins **8a–8d** by introducing different sized cyclic amines on the backbone of luciferin **6**. These new luciferins had similar bioluminescence wavelengths to each other of ~665 nm with PpyWT, but different bio-activities due to their structural variations.^[14] Installation of benzo-fused arenes to the thiazole moiety can improve π -conjugation and benefit nIR emissions. Hall's group synthesized naphthalene luciferin (**9**, OH-NpLH₂) which gave bioluminescence emission at 758 nm with mutant enzyme CBR2 and was applied to bioluminescence tomography in mouse brain.^[15] Branchini and co-workers reported quinoline luciferin (**10**, OH-QLH₂).^[12] With mutant PLR3,^[16] luciferin **10** generated nIR emission at 718 nm. In these latter two examples, although the corresponding linear system is not available for direct comparisons, they both have a similar 'V'-shaped structure similar to **5**, suggesting that this shape and substitution pattern may be beneficial for red shifted emissions.

Design and Synthesis

The bioluminescence of known D-luciferin analogues that have a 'V'-shape benzothiazole motif, seem to correlate with red shifted emission in comparison to more linear structures. We used this simple observation to guide the design of potentially new bioluminescent structures based on the structure of D-luciferin. Conceptually to construct a general 'V'-shaped luciferin (Figure 3b), an aromatic or heterocyclic ring could be fused to the benzothiazole moiety. We also noticed that the benzothiazole core is prevalent in many molecules that possess photophysical properties.^[17] In this work, a novel symmetric 'V'-

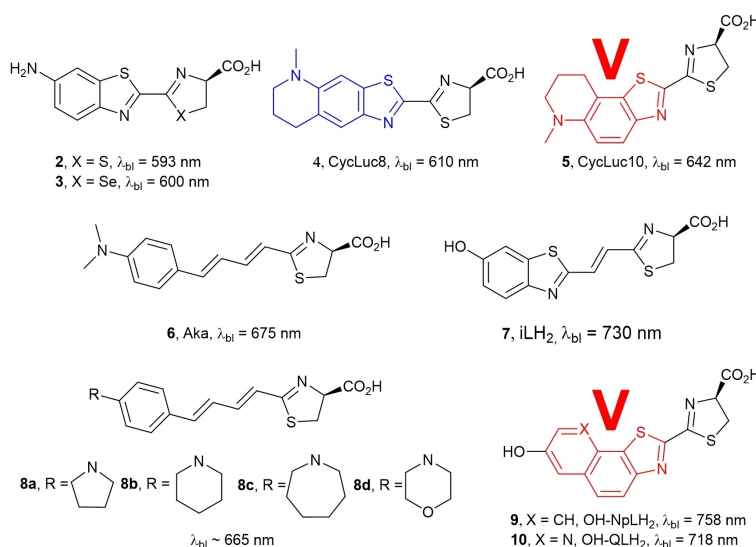


Figure 2. Literature Luciferin analogues with different emission wavelengths.

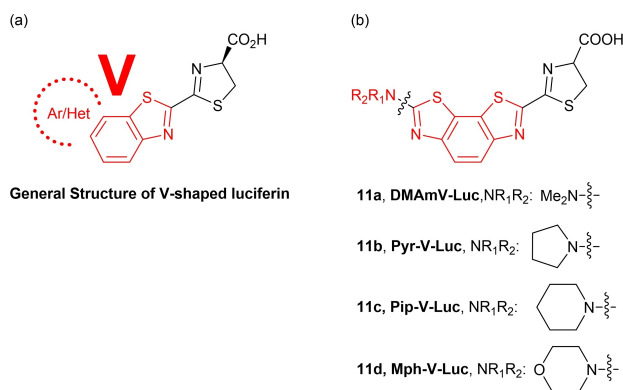
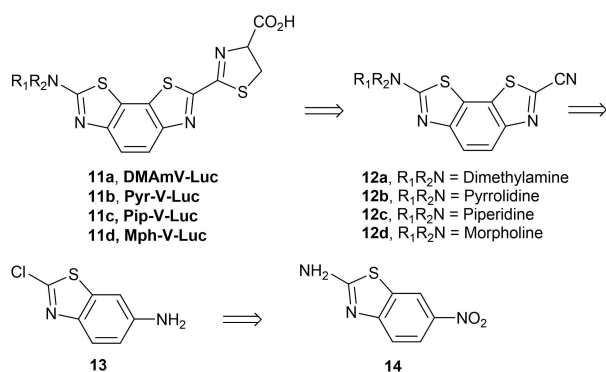


Figure 3. a) General structure of 'V'-shaped luciferin. b) Target 'V'-shaped luciferin analogues.

shaped benzobisthiazole core has been designed and four analogues synthesized that differ in the amino donor substituent **11a–11d** (Figure 3b). A variety of different cyclic amine donors were introduced to construct a push-pull structure and simultaneously investigate possible subtle interactions with amino acid residues in the active site of the luciferase enzyme, which could have an effect on the bioluminescence emission wavelength and activities. Spectroscopic and bioluminescence properties of these new 'V'-shaped analogues were further investigated and compared to D-Luciferin (**1**).

Retrosynthetically the reactive thiazole carboxylic acid ring could be formed by the standard biomimetic condensation of a benzobisthiazole nitrile **12a–d** with cysteine (Scheme 1).^[18] We envisaged that the benzobisthiazole nitriles could be prepared by using the Appel's salt procedure from a requisite aniline.^[19] In this retrosynthetic analysis we were going to investigate the regioselectivity of the formation of the fused thiazole-nitrile with a view to synthesizing both possible isomers for comparison of the linear versus the 'V'-shaped forms.^[20] The desired aniline **13** should already possess a fused thiazole ring with a synthetic handle to enable a late-stage amination reaction. Commercially available 2-amino-6-nitrobenzothiazole (**14**) possessed the requisite functionality to investigate this synthetic strategy (Scheme 1).

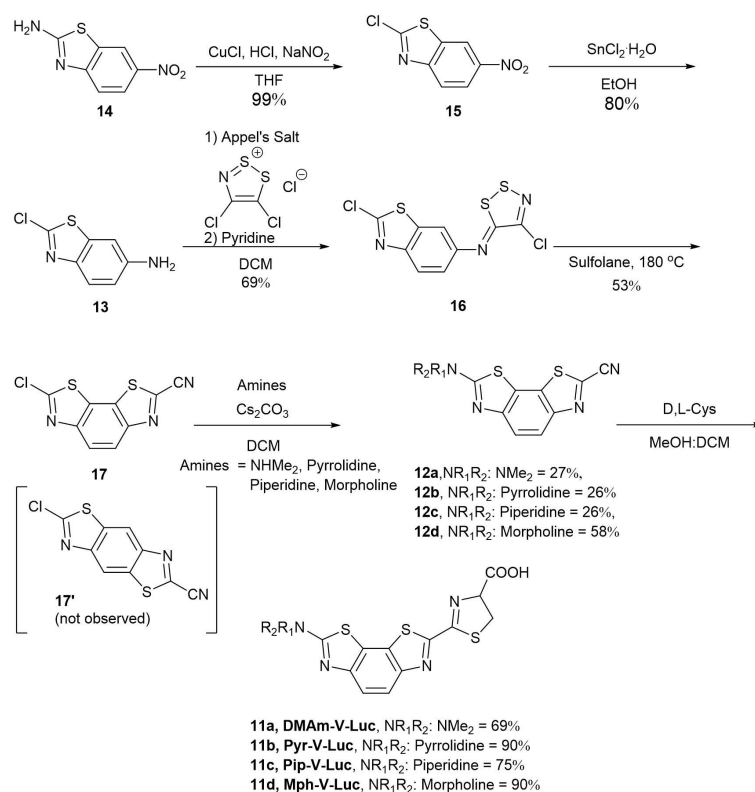


Scheme 1. Retrosynthesis of 'V'-shaped benzobisthiazole luciferin analogues.

Following a literature procedure, **14** was transformed to its corresponding chloro analogue **15** via a Sandmeyer reaction in 99% yield.^[21] The nitro group of **15** was reduced by SnCl_2 in EtOH to give 2-chloro-6-aminobenzothiazole (**13**, 80%),^[8a] which possessed both of the required functionality to construct the target molecules. Condensation of **13** with Appel's salt followed by treatment with pyridine gave the reactive intermediate **16** which was isolated in 69% yield as a single product. The structure of **16** is drawn to represent the reactive isomer required for cyclisation to form the next thiazoline ring, but the geometry of the imine is most likely fluxional. A solution of **16** in sulfolane was heated to 180 °C for 2 h yielding the desired 'V'-shaped benzobisthiazole core structure **17** in 53% yield. The ^1H NMR spectrum of the crude and purified material showed no evidence for the formation of the alternative linear regioisomer **17'**. Reaction of 2-cyano-6-hydroxy-benzothiazole^[22] and D-luciferin^[23] with electrophiles gave major products with substitution at the C7 position of the benzothiazole. This regioselectivity is consistent with the relative stabilities of the Wheland intermediates from initial electrophilic addition. Formation of benzothiazoles from anilines with Appel's salt occurs via an intermediate *N*-arylimino dithiazole. This dithiazole can be considered electrophilic by resonance and drives an electrocyclic ring closure and fragmentation to give the product 2-cyanobenzothiazoles, sulfur and HCl.^[19] The cyclisation of *N*-arylimino-dithiazoles has been shown to be sensitive to predictable substituent effects.^[24] The Claisen rearrangement of 6-allyloxy-2-cyano-benzothiazole gave allylation at the C7 position.^[25] The isolation of **17** as the major and sole product is consistent with the most favourable electrophilic addition to benzothiazole **16** and the formation of the 'V'-shaped isomer is more favourable because it is more aromatic, by virtue of possessing more resonance stabilization energy from both thiazole rings being formally aromatic. Linear isomer **17'** can only ever possess one thiazole ring formally aromatic in any resonance form. A variety of amines were introduced via $\text{S}_{\text{N}}\text{Ar}$ reactions and the corresponding nitrile precursors **12a–d** with different amines (dimethylamine, pyrrolidine, piperidine and morpholine) were provided in modest 27–58% yields. The remaining condensation of nitriles **12a–d** with racemic cysteine in a co-solvent system of DCM/ H_2O (1:1) gave the desired 'V'-shaped luciferin analogues **11a–d** in 69 to 90% isolated yield (Scheme 2).

Analysis of spectroscopic properties

The absorption and fluorescence emission of the 'V'-shaped analogues **11a–d** were investigated and recorded along with D-luciferin (**1**) for comparison. In anhydrous DMSO, the 'V'-shaped analogues exhibited nearly overlapping absorption ranging from 350 nm to 450 nm (Figure 4). Compared to D-LH₂ ($\lambda_{\text{abs}} = 318$ nm), all the 'V'-shaped analogues possessed longer absorption wavelengths: **11a** ($\lambda_{\text{abs}} = 362$ nm), **11b** ($\lambda_{\text{abs}} = 363$ nm), **11c** ($\lambda_{\text{abs}} = 365$ nm), **11d** ($\lambda_{\text{abs}} = 361$ nm), respectively (Table 1). The 'V'-shaped luciferins exhibited similar molar extinction coefficients (ϵ) $\sim 25000 \text{ M}^{-1}\text{cm}^{-1}$ which was 3-fold less than D-LH₂.



Scheme 2. The synthesis of novel V-shaped luciferin analogues.

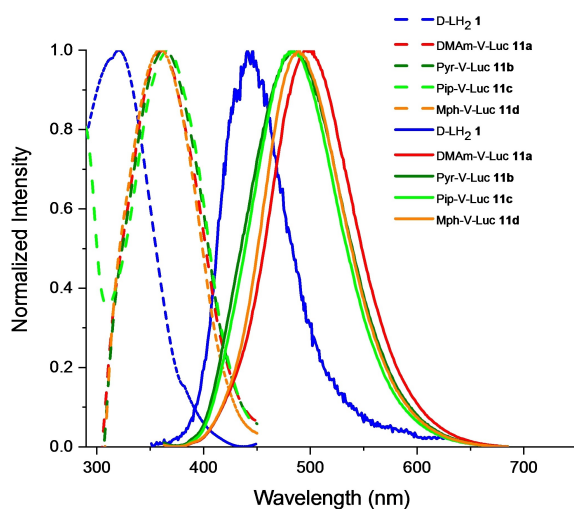


Figure 4. The normalized absorption dash line and fluorescence emission spectra solid line in anhydrous DMSO at r.t..

79200 M⁻¹cm⁻¹. In the fluorescence emission, we observed that all the 'V'-shaped luciferins **11a** ($\lambda_{\text{em}}=501$ nm), **11b** ($\lambda_{\text{em}}=488$ nm), **11c** ($\lambda_{\text{em}}=486$ nm) **11d** ($\lambda_{\text{abs}}=493$ nm) have bathochromic shifted emissions compared to D-LH₂ ($\lambda_{\text{em}}=446$ nm). From the intersection wavelengths (λ_{opt}) of the normalized absorption and emission spectra, the optical band gaps (E_{0-0}) of each luciferin were calculated luciferins **11a–d** have very similar optical band gaps between 2.90 eV to 2.95 eV due to their similar skeletons and D-LH₂ (**1**) has the largest band gap

Table 1. Detailed spectroscopic properties of luciferins.

Luciferins	λ_{abs} [nm]	ϵ [M ⁻¹ cm ⁻¹]	λ_{em} [nm]	E_{0-0} [eV]
1	318	79200	446	3.22
11 a	362	24100	501	2.90
11 b	363	24400	488	2.95
11 c	365	28000	486	2.95
11 d	361	25800	493	2.92

Absorption and fluorescence emission were recorded in anhydrous DMSO solutions at 298 K. The concentrations for absorption and fluorescence emission were 10⁻⁵ M. Optical band gap E_{0-0} was measured from the intersection wavelength (λ_{opt}) of the absorption and emission spectra, $E=1240/\lambda_{\text{opt}}$.

(3.22 eV). The smaller optical band gaps of **11a–d** can be attributed to the extra thiazole ring compared to just the benzothiazole ring in D-LH₂ (**1**). In general, increased aromatic character narrows the band gap and benefits longer wavelength emission. The preliminary optical studies suggested that the luciferins **11a–d** should generate more red-shifted bioluminescence emission than D-LH₂.

Bioluminescence studies

Bioluminescence studies were performed in comparison with D-luciferin (**1**). Initially, the bioluminescence studies were carried out with two types of enzymes *Photinus pyralis* firefly luciferase

(PpyWT) and FlucRed,^[13] a mutant luciferase, both at pH=7.4 and 25 °C. The normalized bioluminescence spectrum of **11a–d** with PpyWT showed a wide range of bioluminescence emission with full width at half maximum (FWHM) above 100 nm (Figure 5a) and about 50% of light emission was in the nIR region. Even though the intensities of these novel analogues **11a–11d** are 95–99.5% weaker than the D-luciferin at their

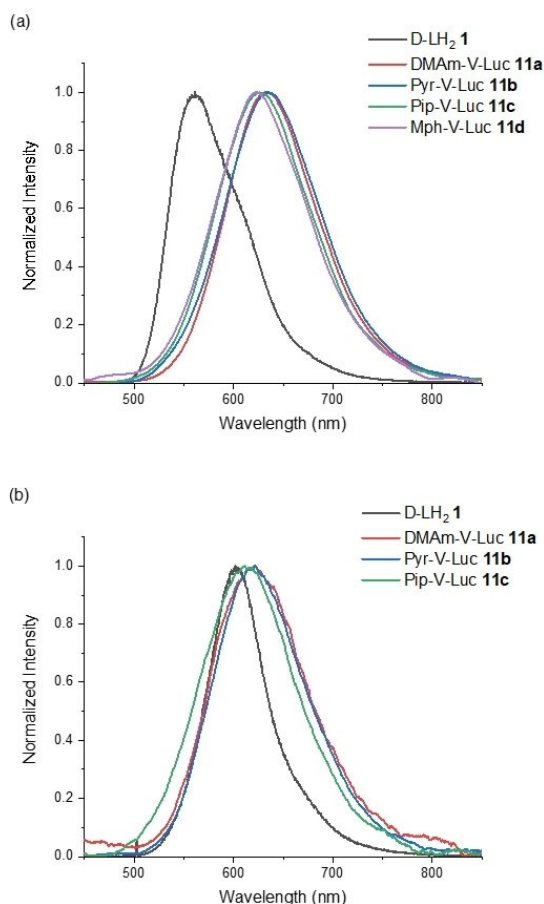


Figure 5. The normalized spectra of luciferins with a) PpyLuc and b) FlucRed.

Table 2. Bioluminescence emission maxima and relative activities with enzyme PpyWT and FlucRed at pH = 7.4.

Luc	Specific activity		BL, 25 °C ($\lambda_{\text{max}} \pm 2$ nm)	
	PpyLuc	FlucRed	PpyLuc	FlucRed
1	100 $\pm$ 4	47.11 $\pm$ 2.6	561 (86)	601 (90)
11a	0.11	0.01	632 (103)	621 (99)
11b	0.18	0.06	636 (113)	620 (108)
11c	0.09	0.01	626 (113)	611 (112)
11d	0.02	0.002	623 (107)	–

Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified enzyme (2.5 μ g), 50 μ M substrate, 1 mM ATP, and 3 mM MgSO₄. Data are expressed as the mean $\pm$ standard deviation and are reported relative to the Luc2/LH₂ value, defined as 100 and were corrected for the spectral response of the CCD detector. Data determined from BL emission spectra measured were obtained as described in the Experimental Section.

λ_{max} 'V'-shaped **11a–d** were able to produce longer emission wavelengths at **11a** (λ_{bl} =632 nm, 97% weaker) **11b** (λ_{bl} =636 nm, 95% weaker) **11c** (λ_{bl} =626 nm, 97% weaker) **11d** (λ_{bl} =623 nm, 99.5% weaker) than D-luciferin (λ_{bl} =561 nm) (Table 2). It is worth noting that the wavelength of **11b** not only gave the most red-shifted emission at 636 nm which is close to the nIR window, but also demonstrated the highest specific activity (0.18) among the four 'V'-shaped luciferins. With mutant FlucRed (Figure 5b), the maximum wavelength of D-luciferin (**1**) was shifted 40 nm from 561 nm to 601 nm. However, luciferins **11a–c** gave the opposite performances. Luciferins **11a–c** provided slightly blue-shifted emissions by ~10 nm, but still more red shifted than D-luciferin, and morpholine **11d** could not generate a proper spectrum due to its weaker specific activity (0.002). The lower specific activities and blue shifted spectrum of 'V'-shaped **11a–d** indicates that the pairs of FlucRed and **11a–d** were less favourable for bioluminescence than the native substrate of PpyLuc with LH₂. Incidentally it has been noted before that FlucRed combined with common aminoluciferins has given slightly blue shifted bioluminescence emissions.^[12]

The 'V'-shaped luciferins **11a** and **11b** were chosen for further bioluminescence investigations with mutant enzymes, CBR2^[12] and PLR3.^[16] This was based on both their red-shifted bioluminescence wavelengths and higher specific activities observed with enzyme PpyLuc (Table 2). The bioluminescence spectra of compounds **11a** and **11b** were similar regardless of whether the enzymes CBR2 or PLR3 were used, and both had wider spectra (FWHM ~100 nm) than D-luciferin **1** (FWHM ~60 nm) (Figure 6). Compared to PpyLuc, both CBR2 and PLR3 showed increased specific activity, but gave slightly blue shifted emission again for the 'V'-shaped analogues as was seen with FlucRed. The highest specific activities were obtained with CBR2, (0.24 and 0.31, respectively) (Table 3) but the intensities at λ_{max} of **11a–b** were still ~95% less than D-luciferin. Although the specific activity of racemic **11a** and **11b** are still lower than enantiomer pure D-luciferin (**1**), **11a** and **11b** showed an approximately 2-fold improvement in activity compared to the wild type enzyme PpyLuc, (0.11 and 0.18 respectively) (Table 2). Meanwhile, D-luciferin (**1**) was half as active with CBR2 (Specific

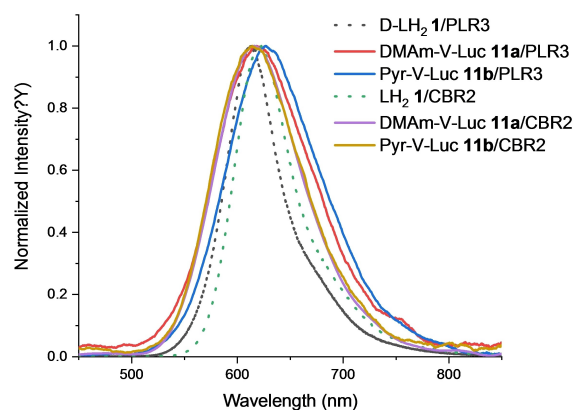


Figure 6. The normalized bioluminescence spectrum of **1**, **11a**, and **11b** with enzymes PLR3 and CBR2.

Table 3. BL emission maxima and relative activities with enzyme CBR2 and PLR3 at pH = 7.

Luc	Specific Activity		BL, 25 °C ($\lambda_{\text{max}} \pm 2$ nm)	
	CBR2	PLR3	CBR2	PLR3
1	52.1 $\pm$ 0.26	74.3 $\pm$ 8	621 (65)	613 (61)
11a	0.24	0.16	616 (95)	618 (105)
11b	0.31	0.21	615 (98)	628 (104)

The methods have been described in Table 2.

Activity = 52.1) in comparison to that with PpyLuc (Specific Activity = 100).

Computational studies

The spectroscopic and bioluminescence studies demonstrated that these extended conjugation amino luciferins exhibited more red-shifted emission than D-luciferin. As our synthetic route did not provide the linear isomer for direct comparison, we undertook TD-DFT studies of the light emitting oxy forms of the 'V'-shaped **18a–d** and linear **19a–d** isomers (Figure 7) to investigate whether the position of ring fusion had an effect on light emission.

The absorption and emission vertical energies were calculated using the TD-DFT method with the CAM-B3LYP functional^[9] and the 6-31G** basis set on QChem5.2.^[26] For vertical energies, the conductor version of PCM (C-PCM) was used to emulate water as the solvent. For their emission energies, the static dielectric constant was used in the C-PCM calculations, considering the solvent in equilibrium with the solute, while perturbative single state (ptSS) and linear response (ptLR) corrections were applied to the zeroth order electronic wavefunction, polarised by the solvent to correct excited state energies. The computational studies showed that the 'V'-shaped

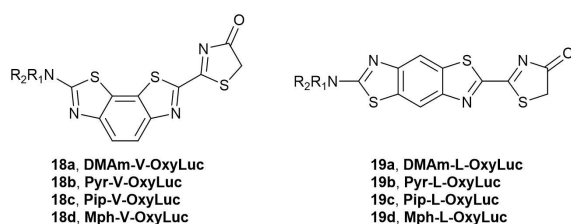


Figure 7. The structures of 'V'-shaped and linear oxyluciferins.

oxy luciferins **18a–d** exhibit smaller vertical transition absorption and emission energies and correspondingly more red-shifted absorption and emission wavelengths than **19a–d** (Table 4). The results demonstrate that the 'V'-shaped structures provide more red-shifted absorptions and emissions than the linear backbones independent of the interaction with luciferases.

Conclusions

In this work, we have created a novel π -extended 'V'-shaped core structure, benzobisthiazole **17**, and used it for the development of 'V'-shaped luciferin analogues **11a–d**. Four structurally and electronically different amines were installed *via* S_NAr reaction, followed by standard condensation with racemic cysteine to give four analogues **11a–d**. It is worth noting that the chloro atom of core **17** potentially provides an opportunity to increase the conjugation by introduction of aromatic rings *via* C–C cross couplings.^[27] Investigation of the new luciferins' spectroscopic properties, showed a more red-shifted fluorescence emissions for the analogues **11a–d**, between 486 nm to 501 nm, than D-luciferin ($\lambda_{\text{em}} = 446$ nm). The corresponding results were also evident in their bioluminescence spectra. With a variety of luciferases, the new luciferins **11a–d** provided longer wavelengths than D-luciferin. The combination of luciferase PpyLuc and Pyr-V-Luc **11b** showed the longest bioluminescence wavelength at 636 nm. Among the 'V'-shaped analogues, luciferin **11b** also had the highest specific activity = 0.18 with PpyLuc and specific activity = 0.31 with mutant CBR2. These novel luciferins **11a–d** also gave a wide range of bioluminescence emission and approximately 50% of their emission was in the nIR region. It is noteworthy that the maximum bioluminescence emissions of dimethylamino luciferin (**2**, with NMe_2 instead of NH_2)^[8a] is similar to that of **11a,b**, and that of CycLuc 10 (**5**) is a little higher,^[8b] despite neither possessing additional π -extension, which has been shown normally to give a large red shifting effect.^[10–12,14–16] The new analogues **11a–d** are also dimmer and therefore give less red light emission than simple amino luciferins and many of Miller's CycLuc analogues. Our computational studies suggested that the 'V'-shaped oxyluciferins **18a–d** have smaller vertical absorption and emission transition energies than the corresponding linear oxyluciferins **19a–d**. The combination of these results illustrates the viability of designing other 'V'-shaped luciferin structures (Figure 3b), based upon an aromatic or heterocyclic ring fused to the benzothiazole moiety of D-

Table 4. Summary of computational results.

V-Oxyluc	$\Delta E_{\text{abs}}/\lambda_{\text{abs}}$ [eV]/[nm]	$\Delta E_{\text{em}}/\lambda_{\text{em}}$ [eV]/[nm]	Linear-Oxyluc	$\Delta E_{\text{abs}}/\lambda_{\text{abs}}$ [eV]/[nm]	$\Delta E_{\text{em}}/\lambda_{\text{em}}$ [eV]/[nm]
18a	3.16/392	3.06/405	19a	3.22/385	3.07/404
18b	3.11/399	3.03/409	19b	3.18/390	3.07/404
18c	3.16/392	3.01/412	19c	3.22/385	3.06/405
18d	3.23/384	3.04/408	19d	3.30/376	3.09/401

luciferin, that opens up a new area for structural investigation of new bioluminescent molecules for red shifted emissions.

Experimental Section

General experimental details – please see Supporting Information.

2-Chloro-6-nitrobenzo[d]thiazole (15). To a solution of 2-amino-6-nitrobenzothiazole (**14**) (1.0 g, 5.1 mmol), CuCl₂ (0.65 g, 4.8 mmol) in MeCN (5 mL) and HCl_(aq) (16 mL) was added NaNO₂ (1.0 g, 15 mmol) slowly at 0 °C and the resulting suspension was stirred overnight at r.t. To consume the starting material completely, the reaction was further heated to 55 °C for 30 min. The solution was then cooled to rt, extracted with CHCl₃ (2×100 mL), and dried (MgSO₄). After filtration, the volatile materials were removed *in vacuo*, to give the crude product that could be used without further purification. The product was obtained as a brown solid (1.1 g, 99%) (lit.^[21] yield 65%). m.p. 187 °C (lit.^[28] 193–194 °C); *R*_f=0.8 (100% CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃) δ 8.75 (1H, s), 8.38 (1H, d, *J*=8.0 Hz), 8.07 (1H, d, *J*=8.0 Hz). The data agreed with the literature.^[21]

2-Chlorobenzo[d]thiazol-6-amine (13). A suspension of 2-chloro-6-nitrobenzo[d]thiazole (**15**) (1.0 g, 5.1 mmol) and tin(II) chloride dihydrate (5.2 g, 23 mmol) in EtOH (30 mL) and HCl_(aq) (5 mL) was refluxed until it turned to a clear brown solution. The volatiles were removed *in vacuo*, and the residue basified to pH=14 with satd. aq. NaOH_(aq), extracted with EtOAc (3×200 mL), and dried (MgSO₄). After filtration, the volatile materials were removed *in vacuo* again, the crude product was further purified column chromatography (silica 50% Hexane:50% EtOAc) to give the desired compound (0.69 g, 80%) (lit.^[8a] yield 77%) as a yellow solid. m.p. 160 °C (lit.^[29] 155–157 °C); *R*_f=0.4 (50% Hexane:50% EtOAc). ¹H NMR (400 MHz, CDCl₃) δ 7.70 (1H, d, *J*=8.7 Hz), 6.99 (1H, d, *J*=2.3 Hz), 6.81 (1H, dd, *J*=8.7, 2.3 Hz), 3.85 (2H, s). The data agreed with the literature.^[8a]

(Z)-4-chloro-N-(2-chlorobenzo[d]thiazol-6-yl)-5H-1,2,3-dithiazol-5-imine (16). To a solution of 2-chlorobenzo[d]thiazol-6-amine **13** (0.23 g, 1.3 mmol) in anhydrous DCM (7 mL) was added Appel's salt (0.29 g, 1.4 mmol), and the mixture stirred at r.t. under N₂ for 2 h. Then pyridine (0.20 mL, 2.5 mmol) was added to the yellow suspension and the reaction stirred for 1 h. The crude reaction mixture was directly purified by column chromatography (silica 50% hexane:50% DCM) to give the desired product **16** as a yellow solid (0.25 g, 63%). m.p. 108 °C (dec.); *R*_f=0.4 (50% hexane:50% EtOAc); IR *v*_{max} (solution in CDCl₃): 3085 (C–H), 3057 (C–H), 2959 (C–H), 2920 (C–H), 2850 (C–H) cm^{−1}. ¹H NMR (700 MHz, CDCl₃) δ 8.02 (1H, d, *J*=8.7 Hz), 7.62 (1H, d, *J*=2.1 Hz), 7.36 (1H, dd, *J*=8.7, 2.1 Hz). ¹³C NMR (175 MHz, CDCl₃) δ 160.0, 153.5, 149.4, 149.1, 148.1, 137.7, 124.4, 119.5, 111.8. HRMS (ESI⁺) Calcd. for C₉H₄Cl₂N₃S₃ [M + H]⁺ 319.8944, found 319.8948.

7-chlorobenzo[1,2-d:4,3-d']bis(thiazole)-2-carbonitrile (17). A solution of (Z)-4-chloro-N-(2-chlorobenzo[d]thiazol-6-yl)-5H-1,2,3-dithiazol-5-imine (**16**) (0.50 g, 1.6 mmol) in sulfolane (3 mL) was heated to 180 °C for 2 h. After cooling to rt, the reaction mixture was purified directly by column chromatography (silica 75% hexane:25% EtOAc) to give product **17** as a yellow solid (0.17 g, 42%). m.p. 212 °C (dec.); *R*_f=0.4 (75% hexane:25% EtOAc); IR *v*_{max} (solution in CDCl₃): 3086 (C–H), 3061 (C–H), 2925 (C–H), (C–H), 2855 (C–H), 2229 (CN) cm^{−1}. ¹H NMR (700 MHz, CDCl₃) δ 8.30 (1H, d, *J*=8.9 Hz), 8.19 (1H, d, *J*=8.9 Hz). ¹³C NMR (175 MHz, CDCl₃) δ 154.4, 151.4, 150.9, 135.9, 128.5, 128.2, 123.9, 123.3, 112.6. HRMS (ESI⁺) Calcd. for C₉H₃ClN₄S₂ [M + H]⁺ 251.9451, found 251.9453.

7-(Dimethylamino)benzo[1,2-d:4,3-d']bis(thiazole)-2-carbonitrile (12a). To a solution of 7-chlorobenzo[1,2-d:4,3-d']bis(thiazole)-2-

carbonitrile (**17**) (50 mg, 0.20 mmol) and Cs₂CO₃ (80 mg, 0.24 mmol) in a co-solvent of DCM (0.5 mL) and water (0.5 mL) was added dimethylamine (2 M in THF) (0.18 mL, 0.36 mmol) at r.t. and then this reaction was stirred overnight. The crude product was purified directly by column chromatography (silica 50% hexane: 50% EtOAc). The desired product **12a** was obtained as a pale yellow solid (17 mg, 27%). m.p. 209 °C (dec.); *R*_f=0.25 (75% hexane:25% EtOAc); IR *v*_{max} (solution in CDCl₃): 2923 (C–H), 2922 (C–H), 2223 (CN) cm^{−1}. ¹H NMR (700 MHz, CDCl₃) δ 8.09 (1H, d, *J*=8.8 Hz), 7.80 (1H, d, *J*=8.8 Hz), 3.28 (6H, s). ¹³C NMR (175 MHz, CDCl₃) δ 169.7 (N–C=N), 154.3 (ArC), 148.0 (ArC), 132.1 (ArC), 128.7 (ArC), 123.1 (ArCH), 121.7 (ArC), 120.1 (ArCH), 113.4 (Ar-CN), 40.6 (N(CH₃)₂). HRMS (ESI⁺) Calcd. for C₁₁H₉N₄S₂ [M + H]⁺ 261.0269, found 261.0271.

7-(Pyrrolidin-1-yl)benzo[1,2-d:4,3-d']bis(thiazole)-2-carbonitrile (12b). Compound **17** (50 mg, 0.20 mmol) was subjected to S_NAr reaction conditions with pyrrolidine (30 mg, 0.36 mmol) as for the preparation of **12a**. After purification by column chromatography (silica 50% hexane:50% EtOAc). The desired product **12b** was obtained as a pale-yellow solid (18 mg, 26%). m.p. 176 °C (dec.); *R*_f=0.25 (50% hexane:50% EtOAc); IR *v*_{max} (solution in CDCl₃): 2949 (C–H), 2922 (C–H), 2872 (C–H), 2852 (C–H), 2221 (CN) cm^{−1}. ¹H NMR (700 MHz, CDCl₃) δ 8.05 (1H, d, *J*=8.8 Hz), 7.78 (1H, d, *J*=8.8 Hz), 3.63 (4H, bs), 2.16–2.12 (4H, m). ¹³C NMR (175 MHz, CDCl₃) δ 166.1, 154.3, 147.8, 131.8, 128.7, 123.0, 121.2, 120.0, 113.4, 50.1, 25.8. HRMS (ESI⁺) Calcd. for C₁₃H₁₁N₄S₂ [M + H]⁺ 287.0425, found 287.0427.

7-(Piperidin-1-yl)benzo[1,2-d:4,3-d']bis(thiazole)-2-carbonitrile (12c). Compound **17** (50 mg, 0.20 mmol) was subjected to S_NAr reaction conditions with piperidine (30 mg, 0.36 mmol) as for the preparation of **12a**. After purification by column chromatography (silica 50% hexane:50% EtOAc), the desired product **12c** was obtained as a pale-yellow solid (18 mg, 26%). m.p. 199 °C (dec.); *R*_f=0.75 (50% hexane:50% EtOAc); IR *v*_{max} (solution in CDCl₃): 2940 (C–H), 2853 (C–H), 2223 (CN) cm^{−1}. ¹H NMR (700 MHz, CDCl₃) δ 8.08 (1H, d, *J*=8.8 Hz), 7.82 (1H, d, *J*=8.8 Hz), 3.72–3.70 (4H, m), 1.78–1.74 (6H, m). ¹³C NMR (175 MHz, CDCl₃) δ 169.3, 148.2, 132.3, 128.6, 123.3, 119.8, 113.3, 77.3, 50.5, 25.5, 24.1. HRMS (ESI⁺) Calcd. for C₁₄H₁₃N₄S₂ [M + H]⁺ 301.0582, found 301.0579.

7-Morpholinobenzo[1,2-d:4,3-d']bis(thiazole)-2-carbonitrile (12d). Compound **17** (50 mg, 0.20 mmol) was subjected to S_NAr reaction conditions with pyrrolidine (30 mg, 0.36 mmol) as for the preparation of **12a**. After purification by column chromatography (silica 50% hexane:50% EtOAc). The desired product **12d** was obtained as a pale-yellow solid (40 mg, 58%). m.p. 179 °C (dec.); *R*_f=0.8 (50% hexane:50% EtOAc); IR *v*_{max} (solution in CDCl₃): 2957 (C–H), 2918 (C–H), 2848 (C–H), 2221 (CN) cm^{−1}. ¹H NMR (700 MHz, CDCl₃) δ 8.11 (1H, d, *J*=8.9 Hz), 7.80 (1H, d, *J*=8.9 Hz), 3.88–3.84 (4H, m), 3.71–3.69 (4H, m). ¹³C NMR (175 MHz, CDCl₃) δ 169.7, 153.2, 148.4, 132.7, 128.7, 123.3, 121.1, 120.4, 113.3, 66.3, 48.9. HRMS (ESI⁺) Calcd. for C₁₃H₁₁N₄OS₂ [M + H]⁺ 303.0369, found 303.0369.

2-(7-(Dimethylamino)benzo[1,2-d:4,3-d']bis(thiazole)-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (11a). A solution of 7-(dimethylamino)benzo[1,2-d:4,3-d']bis(thiazole)-2-carbonitrile (**12a**) (10 mg, 0.030 mmol), triethylamine (8.0 mg, 10 μL, 0.060 mmol) and D,L-cysteine (50 mg, 0.040 mmol) in DCM (0.5 mL) and water (0.5 mL) was stirred at rt under N₂ for 2 days. After the solvents were removed *in vacuo*, the crude product was further sonicated with H₂O (1 mL) and CHCl₃ (1 mL). After filtration, the desired product **11a** was collected as a yellow solid (15 mg, 69%). m.p. 166 °C (dec.); IR *v*_{max} (solid): 3394 (COOH), 3041 (C–H), 2918 (C–H), 1709 (C=O) cm^{−1}. ¹H NMR (700 MHz, Methanol-*d*₄) δ 7.97 (1H, d, *J*=8.8), 7.65 (1H, d, *J*=8.8), 5.25 (app t, *J*=9.3, 1H), 3.76 (2H, dd, *J*=9.3, 7.4), 3.26 (6H, s). ¹³C NMR (175 MHz, Methanol-*d*₄) δ 171.0, 159.4,

153.8, 149.9, 129.8, 123.2, 122.8, 119.3, 82.4, 40.7, 37.2. HRMS (ESI⁺) Calcd. for C₁₄H₁₃N₄O₂S₃ [M+H]⁺ 365.0195, found 365.0184.

2-(7-(Pyrrolidin-1-yl)benzo[1,2-d:4,3-d']bis(thiazole)-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (11b). Compound **12b** (10 mg, 0.030 mmol) was subjected to condensation conditions as for the preparation of **11a**. The desired product **11b** was obtained as a yellow solid (12 mg, 90%). m.p. 183 °C (dec.); IR $\nu_{\max}$ (solid): 3378 (COOH), 2925 (C–H), 2925 (C–H), 2825 (C–H), 1727 (C=O) cm^{−1}. ¹H NMR (700 MHz, Methanol-*d*₄) δ 7.98 (1H, d, *J*=8.7 Hz), 7.66 (1H, d, *J*=8.7 Hz), 5.26 (1H, app t, *J*=9.2 Hz), 3.75 (2H, dd, *J*=9.2, 7.5 Hz), 3.61 (4H, bs), 2.14 (4H, bs). ¹³C NMR (175 MHz, Methanol-*d*₄) δ 177.4, 167.5, 165.0, 159.7, 153.7, 149.9, 130.0, 123.1, 122.3, 119.0, 83.2, 51.0, 37.5, 26.7. HRMS (ESI⁺) Calcd. for C₁₆H₁₅N₄O₂S₃ [M+H]⁺ 391.0352, found 391.0350.

2-(7-(Piperidin-1-yl)benzo[1,2-d:4,3-d']bis(thiazole)-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (11c). Compound **12c** (10 mg, 0.030 mmol) was subjected to condensation conditions as for the preparation of **11a**. The desired product **11c** was obtained as a yellow solid (10 mg, 75%). m.p. 189 °C (dec.); IR $\nu_{\max}$ (solid): 3379 (COOH), 2930 (C–H), 2850 (C–H), 1740 (C=O) cm^{−1}. ¹H NMR (700 MHz, Methanol-*d*₄) δ 7.96 (1H, d, *J*=8.8 Hz), 7.63 (1H, d, *J*=8.8 Hz), 5.20 (1H, app t, *J*=9.3 Hz), 3.73 (2H, dd, *J*=9.3, 6.6 Hz), 3.68 (4H, bs), 1.75 (6H, bs). ¹³C NMR (175 MHz, Methanol-*d*₄) δ 177.4, 170.8, 165.0, 159.7, 153.6, 150.1, 129.8, 123.0, 122.5, 119.3, 83.2, 51.1, 37.5, 26.4, 25.1. HRMS (ESI⁺) Calcd. for C₁₇H₁₆N₄O₂S₃ [M+H]⁺ 405.0508, found 405.0514.

2-(7-Morpholinobenzo[1,2-d:4,3-d']bis(thiazole)-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (11d). Compound **12d** (10 mg, 0.030 mmol) was subjected to condensation conditions as for the preparation of **11a**. The desired product **11d** was obtained as a yellow solid (12 mg, 90%). m.p. 164 °C (dec.); IR $\nu_{\max}$ (solid): 3343 (COOH), 2922 (C–H), 2853 (C–H) cm^{−1}. ¹H NMR (700 MHz, Methanol-*d*₄) δ 7.99 (1H, d, *J*=8.8 Hz), 7.68 (1H, d, *J*=8.8 Hz), 5.23 (1H, app t, *J*=9.2 Hz), 3.86–3.83 (4H, m), 3.75 (2H, dd, *J*=9.3, 7.5 Hz), 3.70–3.68 (4H, m). ¹³C NMR (175 MHz, Methanol-*d*₄) δ 177.1, 171.2, 160.0, 153.3, 150.4, 130.0, 126.3, 123.1, 122.8, 119.8, 83.0, 67.2, 49.9, 37.4. HRMS (ESI⁺) Calcd. for C₁₆H₁₅N₄O₃S₃ [M+H]⁺ 407.0301, found 407.0309.

Bioluminescence (BL) emission spectra^[12]

BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of assay buffer containing 12.5 μ g of enzyme and 0.1 mM of LH₂ and the 'V'-shaped luciferins **11a–d** in a quartz cuvette. All solutions were pre-warmed to 25 °C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 25 °C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450 nm to 925 nm, with the excitation source turned off and the emission slit width set to 10 nm, and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer. Assay pH values were confirmed before and after spectra were obtained.

Specific activities

All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP, and 6 mM MgSO₄. Signals were monitored over 2 min using a

Synergy™ 2 microplate luminometer (BioTek, Winooski, VT). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

Computational studies

The density functional theory (DFT) method was chosen to optimise the electronic ground state of the **18a–d** and **19a–d** with the global hybrid CAM-B3LYP functional. We performed optimisations with the 6-31G** basis sets to decrease the calculation time and expense. The calculation methods for vertical transition energies with TD-CAM-B3LYP with PCM solvent were detailed in Computational Studies section in the main text. The simulations were carried out with QChem5.2.

Supporting Information

The authors have cited additional references within the Supporting Information.^[28,29]

Author Contributions

C.-H.C. and J.C.A. designed the structures. J.C.A. supervised the project. C.-H.C. performed the synthesis and spectroscopic measurement. D.F. and B.R.B. determined the bioluminescence data and interpreted the results. S.G. conducted the computational calculations, S.G. and C.-H.C. analysed the results. All authors contributed to writing the paper.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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