

Synthesis and evaluation of D-thioluciferin, a bioluminescent 6'-thio analog of D-luciferin

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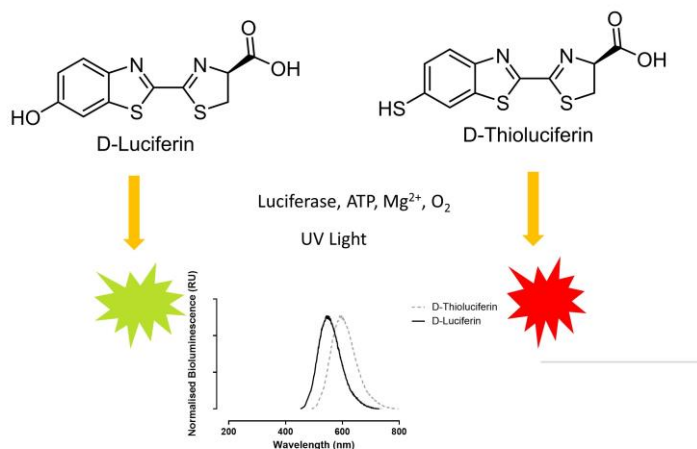
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Abstract

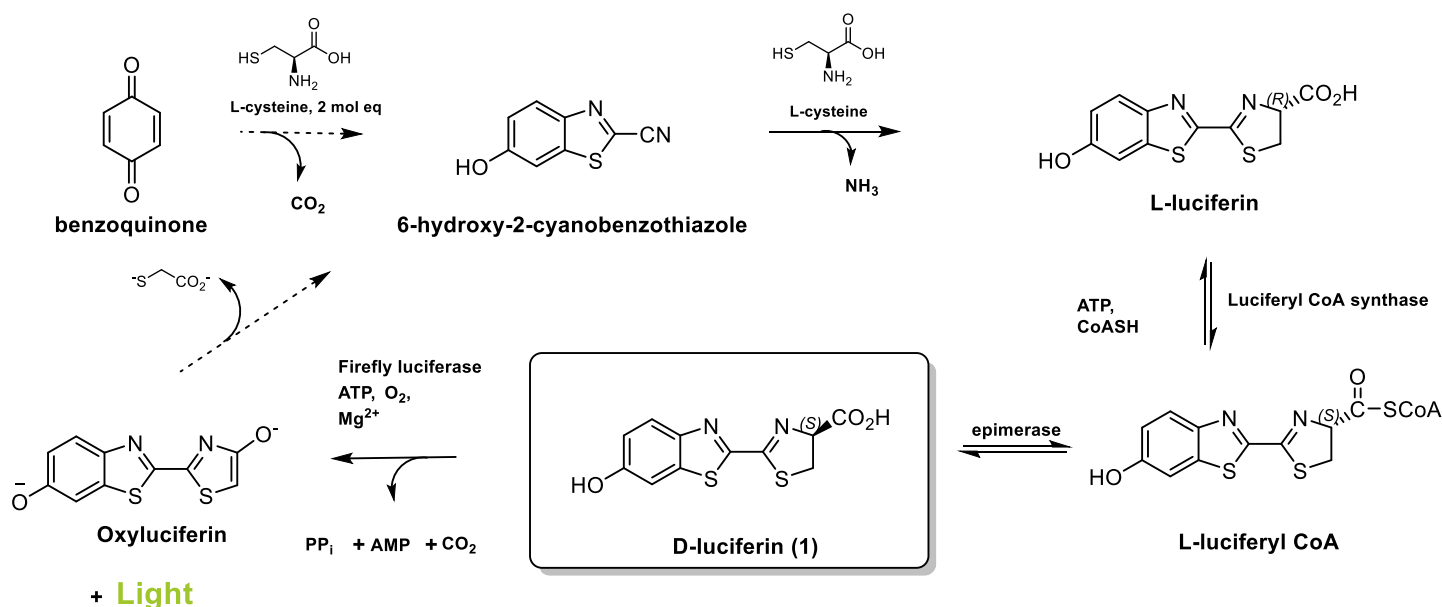
All known light-emitting firefly-bioluminescent luciferin analogs are either derived from the 6'-hydroxy- and/or 6'-aminoluciferin. We report the synthesis of D-thioluciferin, a 6'-thio analog or isostere of D-luciferin, starting from *p*-aminothiophenol, using a unique thioacrylate-S-protecting-group strategy. Upon treatment of D-thioluciferin with purified *Photinus pyralis* (Ppy) luciferase (Luc), a bioluminescence emission with a red-shift λ_{\max} relative to D-luciferin was observed. It was also shown that disulphide and sulphide analogs of D-thioluciferin did not produce similar bioluminescences relative to D-thioluciferin when treated with Ppy Luc under standard conditions, thus, providing a foundation for the development of D-thioluciferin based probes based on disulphide reduction and S-dealkylation.



Keywords: Bioluminescence, D-luciferin, D-thioluciferin, firefly luciferase

Introduction

D-Luciferin (**1**) is the light-emitting molecule responsible for the bioluminescence observed in the American firefly *Photinus pyralis* (scheme 1).¹ Biosynthesis of D-L-luciferin starts with quinone followed by the addition of two mol equivalents of L-cysteine with concomitant loss of CO₂.² The enzyme-controlled stereochemical inversion of L-luciferin to D-luciferin occurs by activation with CoA by virtue of a thioester conjugate at the carboxyl moiety.³ The latter process can be considered a natural light switch that is utilised as a means of communication for the firefly. Both D- and L-luciferin reacts in the presence of O₂, ATP, Mg²⁺ and the luciferase enzyme, but only D-luciferin (**1**) produces a yellow/green light. There is speculation that the firefly does not waste the resulting oxyluciferin and can use luciferin-regenerating enzyme (LRE) to produce the 6-hydroxy-1,3-benzothiazole-2-carbonitrile once again. How, or even if, the oxyluciferin is recycled, is still under investigation. The chirality at the carboxyl group in natural firefly luciferin is of the *S* form, as it was established by the early chemical synthesis of D-luciferin (**1**) from 6-hydroxy-1,3-benzothiazole-2-carbonitrile and D-cysteine. L-Luciferin has the *R* form and is not used for the luminescence reaction by firefly luciferase.⁴ Thus, firefly luciferase oxidizes only D-luciferin, a specificity which has been exploited in gene reporter as well as cell viability assays based on ATP production.⁵



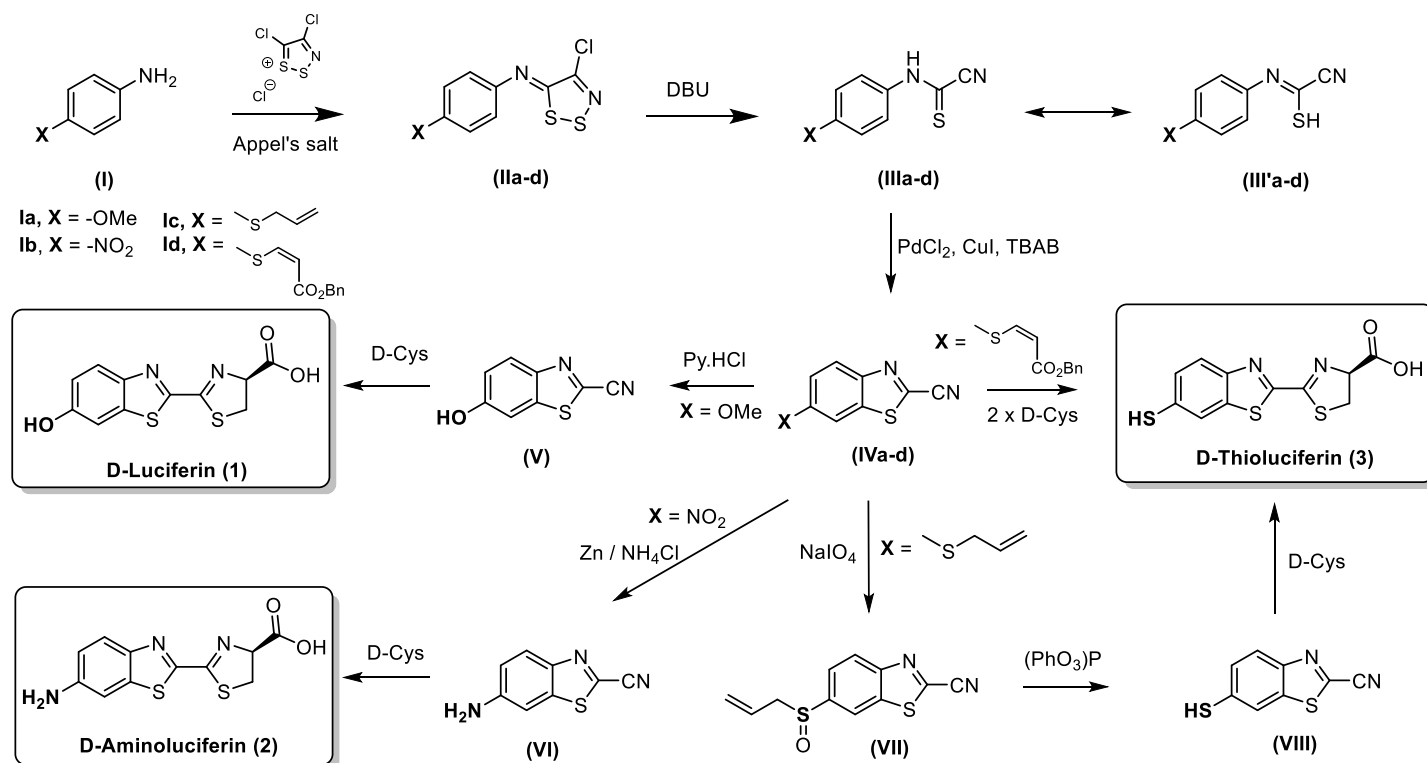
Scheme 1. Biosynthesis of D-luciferin (**1**) in the firefly, *Photinus pyralis*.

Modifications to the natural substrate have resulted in new luminogenic substrates with often improved properties, which have been exploited in the development of sensitive luciferin-based probes for in-vivo imaging, also known as “caged luciferins”.⁶ Most of these probes, however, are based on the release of either natural D-luciferin (**1**) or a 6'-amino analog, D-6-aminoluciferin (**2**).^{7, 8} The natural 6-OH and synthetic 6-NH₂ bioluminescent substrates have limited bioanalytical applications, particularly in terms of coupling bioluminescence activity directly with sulfur biology. The development of thiol-sensing technologies has recently become an area of increased interest because of the biological importance of thiol-containing molecules such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH).⁹ Most of these technologies involve fluorescent or colorimetric quantitation, despite bioluminescence being reportedly more sensitive.¹⁰⁻¹¹

It is known that $\text{HC}\equiv\text{C}$ -EWG compounds with strong EWGs such as SO_2R and CO_2R can react at ambient temperatures with relatively very weak nucleophiles, in the presence of suitable catalysts.¹² In the latter report, pyrrolidine-mediated deprotection of thiolacrylates has been demonstrated in organic medium. The reactions of Cys with $\text{HC}\equiv\text{C}$ -CONHR, which are poorer Michael acceptors, proved to be sufficiently quick, complete, and Z-stereoselective in aqueous media.¹³ The kinetics of thioacrylate protection of cysteine and its release under physiological conditions have been investigated.¹⁴ Notably, peptides modified by terminal alkynones could be converted back into the unmodified peptides by treatment with thiophenol and free cysteine under mild reaction conditions.

With the aim of creating a bioluminescent molecule that would be well suited for applications in biological sulfur chemistry, a 6'-thio analog of firefly luciferin, D-thioluciferin (**3**), was envisaged (Scheme 2). Sharma et al. reported the synthesis of 6-fluoro-1,3-benzothiazole-2-carbonitrile, and attempted $\text{S}_\text{N}\text{Ar}$ reactions with a variety of cyclic secondary amines, including MeSH and ArSH, which resulted in very low yields and no feasible thiol-deprotection method.¹⁵ Existing methods of producing D-luciferin typically start with construction of a 6-hydroxy-1,3-benzothiazole-2-carbonitrile (**V**) intermediate, and require over 7 synthetic steps which result in low overall yields. The synthesis of D-luciferin (**1**) was achieved through the condensation of 4,5-dichloro-1,2,3-dithiazolium chloride (Appel's salt) with *p*-anisidine (**1a**), generating an *N*-arylimino-1,2,3-dithiazole (**IIa**), which then underwent DBU-mediated dithiazole fragmentation to afford the corresponding cyanothioformanilide (**IIIa**). The latter intermediate resonates with its thione form (**IIIa'**). A copper iodide/palladium chloride-catalysed C-S cross-coupling reaction was then used to form the 6-methoxy-1,3-benzothiazole-2-carbonitrile (**IVa**) from the thioacetamide precursor. Subsequent *O*-alkyl deprotection generated the key 6-hydroxy-1,3-benzothiazole-2-carbonitrile (**V**). Facile D-Cys addition on the nitrile functionality of 6-hydroxy-1,3-benzothiazole-2-carbonitrile (**V**) generated the carboxy-thiazoline ring of D-luciferin (**1**) with the correct stereochemistry required for luminescent activity. Likewise, 6-aminoluciferin (**2**) was prepared by substituting the *p*-anisidine (**1a**) with *p*-nitroaniline (**1b**) that required a reductive step to give the intermediate 6-amino-1,3-benzothiazole-2-carbonitrile (**VI**). The methoxy and nitro functionalities served as protected or surrogate functional groups that were transformed, after 1,3-benzothiazole-2-carbonitrile formation, to afford the desired 6'-hydroxy or amino groups. Zinc-mediated reduction of the 6-nitro-1,3-benzothiazole-2-carbonitrile (**IVb**) gave 6-amino-1,3-benzothiazole-2-carbonitrile (**VI**) which was readily converted to D-aminoluciferin (**2**) by the addition of D-cysteine.

Using the latter synthetic approach requires a *p*-aminothiophenol with an ideal sulfur-protecting group that could withstand the subsequent reaction conditions. Moreover, preparation of luciferin derivatives requires the use of a palladium (II) catalyst, which is expensive to use and can be poisoned by free thiol-containing reagents. There is, therefore, a need for novel synthetic methods of producing D-thioluciferins and their derivatives. We utilised an expedient synthetic approach for D-thioluciferin (**3**) (Jardine et al. PCT/IB2018/055542) based on the established preparation of both D-luciferin (**1**) and D-aminoluciferin (**2**).⁸ Most thioether-sulfur protecting groups are either too labile (e.g., trityl) or too stable (e.g., alkyl) for consideration under the latter synthetic strategy.¹⁶ In addition, thioesters could not be considered as protecting groups since the cross-coupling reaction that produces benzothiazoles from thioanilides also produces benzothiofurans. More recently, Pirrung et al. reported the synthesis of D-thioluciferin (**3**) based on the Mislow-Evans rearrangement.¹⁷⁻¹⁸ This methodology proceeds via the 6-allylthiobenzothiazole (**IV**), followed by periodate oxidation, to give the allylic sulfoxide (**VII**), which then rearranges to an intermediate allylic sulfenate that is subsequently cleaved by triphenyl phosphite, as a reductant, to give the target intermediate 6-mercapto-1,3-benzothiazole-2-carbonitrile (**VIII**). Facile addition of D-cysteine gave D-thioluciferin (**3**).



Scheme 2. Synthesis of D-Luciferin (**1**)⁸, D-Aminoluciferin (**2**)⁸ and D-Thioluciferin (**3**).¹⁷

Results and Discussion

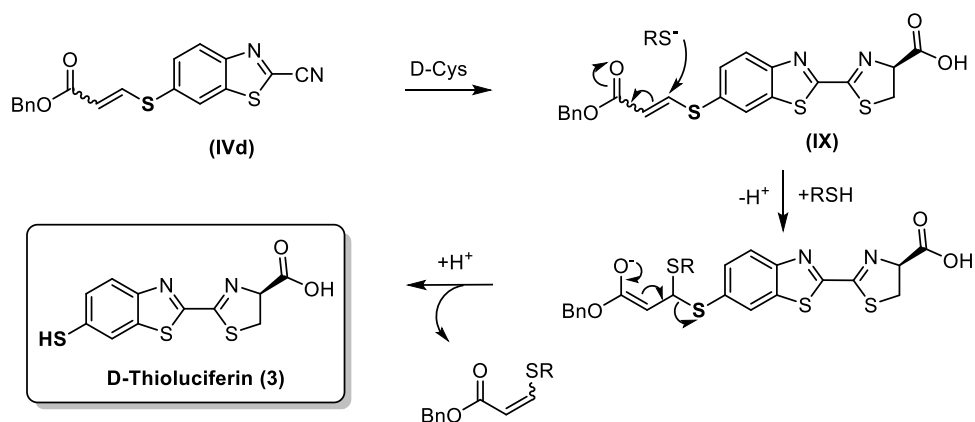
Synthesis

As protection for the thiol group, the aryl thioacrylate (**Id**) was prepared with the premise that it would be stable to subsequent reactions (Scheme 2). Thus, using well known thiol-click (thiol-yne)-type chemistry between benzyl propiolate and *p*-aminothiophenol, the aryl thioacrylate (**Id**) was obtained in a chemoselective manner. The thiol oxidation gave the 4,4'-disulfanediyl dianiline (disulfide) as a byproduct which could be recycled. The benzyl-propiolate ester was used instead of the free acid to prevent the undesired quenching of basic DBU in a subsequent step. The reaction of the propiolate ester with *p*-aminothiophenol produced the aryl thioacrylate (**Id**) as a 3:7 (*E*:*Z*) mixture of geometric isomers. The *Z*-isomer was isolated in 39% yield while the remaining fractions were a mixture of *E*:*Z* isomers. Thiol addition to the acetylenic carbon is *anti* as expected, and the resulting thioacrylate was *Z*-selective. The *Z*-selectivity agrees with work done by Crisp and Millan, and Arjona *et al.* in which they used conjugated alkynes as protecting agents for thiols.¹⁹⁻²⁰ The reaction stereoselectivity was tunable under variable temperatures, solvents and the base, but was not further optimised.

Established Appel synthetic methodology provided the *N*-aryliminodithiazole (**IId**), after Appel's salt condensation, in 99% yield. Subsequent conversion of the *N*-aryliminodithiazole (**IId**) to the cyanoformanilide (**IIId**) in 61% yield was achieved using the thiophilic nucleophile DBU. The cyanoformanilide (**IIId**) was found to be in equilibrium with its thione tautomeric form (**III'd**). Subsequent palladium-chloride-mediated intramolecular C-S coupling furnished the cyclised 6-[benzyl-(*E/Z*)-3'-mercaptoacrylate]-1,3-benzothiazole-2-carbonitrile (**IVd**) in 62% yield. It was also envisaged that the thioacrylate deprotection of carbonitrile (**IVd**)

would release D-thioluciferin (**3**) via the addition of 2 mol equivalents D-cysteine. The thiazoline ring formation is known to be a facile, near quantitative, reaction. The regioselective addition of one mol of cysteine to complete the carboxy-thiazoline ring to give the thioacrylate protected thioluciferin (**IX**) and, subsequently, another mol equivalent of D-cysteine to effect a thia-Michael addition, followed by a retro-Michael reaction, resulting in the release of D-thioluciferin (**3**). Gratifyingly, the thioacrylate (**IVd**) was well tolerated by the coupling reaction, which was interesting because there are not many reported options for thiol-protecting groups which are both easily removed and stable to palladium-mediated chemistry.

The unsaturated vinyl sulfide units (**IVd** or **IX**) could be cleaved by an addition/elimination mechanism by treatment with a thiol (RSH) (Scheme 3). Notably, D-cysteine reacts regioselectively at the nitrile group when limited to 1 mol equivalent. Thiol-sensitive or “caged” D-thioluciferin (**IX**) could essentially be deprotected with any biothiol (RSH) in aqueous medium. Shiu et al. investigated the modification of cysteine-containing peptides in which they found that thiol-protection of cysteine using electron-deficient alkynes favoured formation of the Z-isomer.¹⁴ Accordingly, peptides modified by terminal alkynones could be converted back into the unmodified peptides by treatment with thiols under mild reaction conditions. The driving force for the reaction is the elimination of the more stable thiolate anion of D-thioluciferin.



Scheme 3. Mechanism of thiol mediated thioacrylate (**IX**) deprotection and simultaneous D-thioluciferin (**3**) synthesis.

Spectroscopic characterization

In the presence of ATP, D-luciferin (**1**) is oxidized by luciferase to generate oxyluciferin, thereby, resulting in production of bioluminescence and loss of fluorescence proportional to the concentration of ATP. The emission wavelength of bioluminescence for D-thioluciferin (**3**) was then evaluated (Figure 1). It exhibited a red-shifted light emission (599 nm) when treated with purified firefly luciferase (luc) expressed from *E. coli*, relative to (**1**) (557 nm) and D-aminoluciferin (**2**) (593 nm)

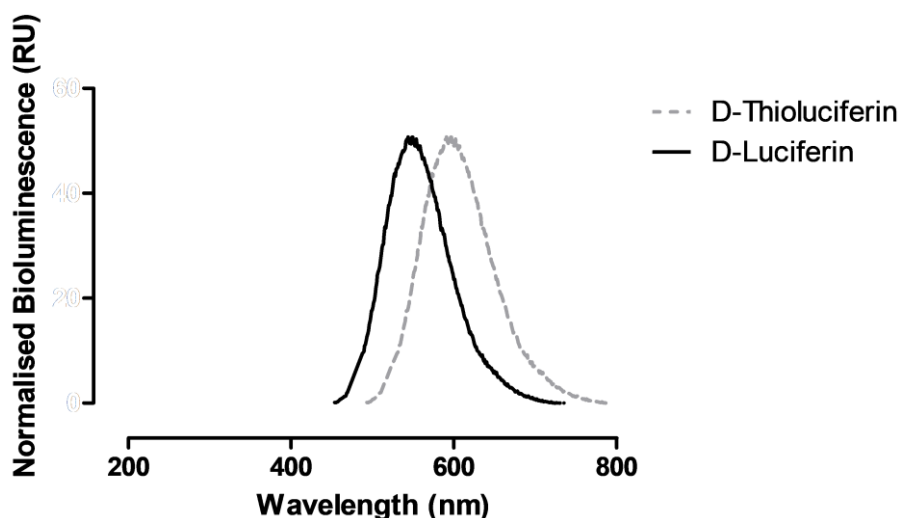


Figure 1. Normalised bioluminescence emission spectra of 100 μ M D-luciferin and 100 μ M D-thioluciferin treated with 10 nM luciferase.

The efficiency of a bioluminescent reaction is determined by the product of the quantum yield and reaction rate. Thus, quantitative analysis and knowledge of the quantum yield and the reaction kinetics are important. The emission intensity increased, as expected, with increasing concentrations of (**3**) when treated with purified luciferase under standard conditions (Figure 2a). No emission was observed for the pure enzyme in the absence of D-luciferin (**1**) (control 1) as well as for the pure substrate in the absence of the enzyme (control 2). The burst-kinetics profile of (**3**) (Figure 2b) was like that reported for both (**1**) and (**2**). A rapid-injection experiment was performed in which the light output for the reaction over time was recorded. As with all known luciferins, (**3**) gave a robust initial burst of light followed by sustained light output of much lower intensity (Figure 2b). This trend is consistent with that previously reported for (**1**) and (**2**) where rapid decay in emission intensity post-burst corresponds to product inhibition.²¹ The lower emission intensity of (**3**) relative to the natural substrate (**1**) should, however, not be a deterrent for its applications in bioluminescence imaging. Such applications rely purely on light generated from the enzyme-substrate reaction and, as a result, generally have good sensitivity. Notably, (**3**) displayed >100-fold emission over the background.

The relative luminescence-emission intensity of natural (**1**) (Figure 2c) was, however, 100-fold greater than both (**2**) and (**3**) when treated with purified firefly luciferase as compared with the corresponding negative controls (substrates in the absence of luciferase) (Figure 2d). As reported for (**2**), (**3**) was also found to have a 100-fold less intense emission signal when compared to (**1**). The reduction in light output could be due to substrate (**3**) combined with a luciferase light-emitting reaction having a lower quantum yield or because of differences in the rate of oxyluciferin production.

To lay the groundwork for biothiol-specific biosensing applications, the thioacrylate sulphide (**IX**) and the D-thioluciferin homodisulphide (**3'**) (Figure 2e), prepared from an iodine oxidation, were also evaluated for the bioluminescent reaction. Neither produced a bioluminescent signal comparable to D-thioluciferin (**3**).

Importantly, for the purpose of thiol sensing, the luminescence output for (**3**) was 90-fold greater than its *S*-protected-thioacrylate (**IX**), and 2.5-fold greater than the D-thioluciferin homodisulphide (**3'**), when treated with luciferase under physiological conditions (Figure 2d). It was demonstrated that neither pure luciferase nor pure D-thioluciferin thioacrylate (**IX**, control 1) emitted light. It was also demonstrated that,

when a 0.1 μ M thioacrylate solution was treated with luciferase in enzyme buffer, the luminescence output remained negligible. This reinforces that the thioacrylate (**IX**) of (**3**) is, indeed, not a substrate for luciferase-mediated bioluminescence, and, perhaps by extension, that all sulphides of (**3**) are inactive, as is the case with (**1**) and its 6'-O-alkyl analogues and the previously reported D-luciferin-6'-sulphides.¹⁵ Negative controls (Figure 2d) contained the homodisulphide (**3'**) and (**3**), respectively. In both cases, in the absence of luciferase, a small degree of luminescence was detected (4% above background). The luminescence increased significantly when (**3**) and its disulphide were treated with luciferase to a final enzyme concentration of 10 nM. Notably, the homodisulphide (**3'**) treated with luciferase emitted a degree of bioluminescence relative to the corresponding control. This effect can be ascribed to the reduction of the non-bioluminescent D-thioluciferin homodisulphide (**3'**) to the bioluminescence-active-free (**3**) by the reducing agent in the enzyme buffer, namely DTT. Kinetic data of the D-thioluciferin homodisulphide (**3'**), however, did not show an increase in bioluminescence over time as one would expect if the free thiol were constantly being formed via disulphide reduction. Instead, the degree of bioluminescence was observed to be decreasing over time, and the rate of decrease in bioluminescence was comparable to that of (**3**). This could indicate that the reduction with DTT had occurred relatively quickly, generating a fixed amount of (**3**) which was not replenished via further disulphide reduction. As a result, the free thiol displayed five-fold greater luminescence than the disulphide, which is indeed a promising result for future redox based sensing applications.

From the kinetic assays, it was also observed that D-thioluciferin (**3**)'s rate of decay in bioluminescence emission, when treated with luciferase under standard conditions, was reduced when compared to that of D-luciferin (**1**) and D-aminoluciferin (**2**). This was a particularly attractive discovery since luciferins are generally known not to have a very stable bioluminescence output and, therefore, require constant re-supply or re-administration. These bioluminescent properties are a good starting point for D-thioluciferin (**3**)-based bioluminescence imaging, despite the lower bioluminescence output relative to D-luciferin (**1**).

It has been reported that size and hydrophobicity at the C-6 position influence the quantum yield of cyclic amino-luciferins.²² Other factors include pH and the microenvironment in the enzyme active site. Substitution of the 6'-oxygen in D-luciferin (**1**) with a nitrogen or sulphur resulted in a weakening of bioluminescent intensity, a phenomenon that is not fully understood yet, but might involve bivalent metal ions, which are a cofactor in enzymatic reactions of firefly bioluminescence.

Interestingly, the 6'-methylthio-luciferin reported by Miller et al.¹⁵ proved not to be a substrate for luciferase, whereas 6'-N-alkylated-aminoluciferins were better in vivo substrates for bioluminescence experiments than (**2**) itself.²³ The absorbance of the 6'-methylthio-luciferin is slightly red-shifted compared to (**1**), which is opposite to the trend observed with (**3**). The fluorescence of the S-methyl-thioluciferin is blue-shifted ca. 40 nm, and there was a reduction of the fluorescence quantum yield. In addition to its bioluminescent emission, however, (**3**) was found to have a strong fluorescence emission, while its protected thioacrylate (**IX**) was only weakly fluorescent, after excitation at a range of wavelengths (360-520 nm) (Figure S1-S5). Thus, the latter molecules provide further opportunities for imaging applications, the most obvious of which relate to sulphide deprotection and disulphide reduction.

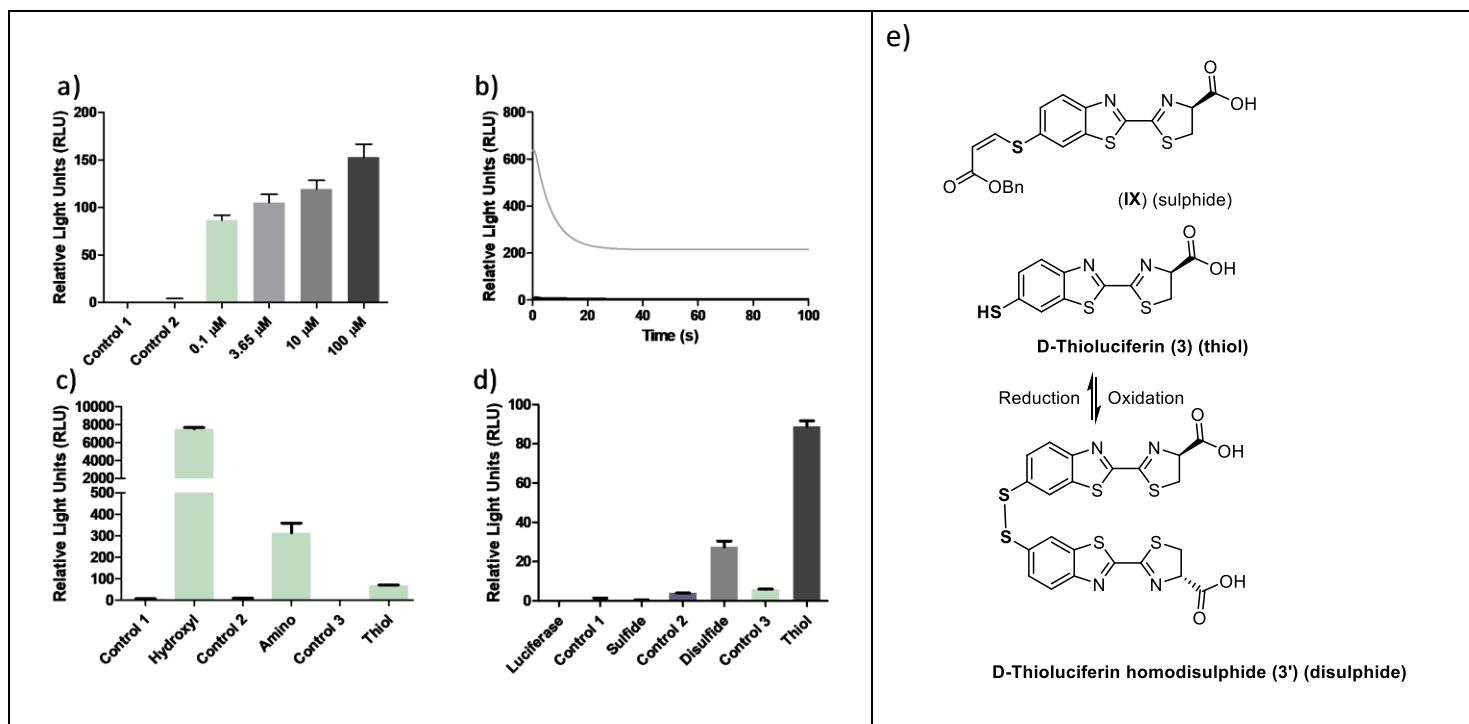


Figure 2. a) Graph of luciferase luminescence at a final enzyme concentration of 10 nM, at varying D thioluciferin concentrations 1 min post-enzyme addition (control 1 is the emission recorded for the enzyme solution in the absence of substrate (1) and control 2 is the recorded emission for substrate (2) in the absence of the enzyme). b) Burst kinetics profile of purified 10 nM luciferase treated with 100 μ M D thioluciferin. c) Relative luminescence emission intensity of the core luciferins (6-hydroxyl, 6-amino and 6-thiol at 0.1 μ M substrate concentration and at a final luciferase concentration of 10 nM. Control 3 is the recorded emission for substrate (3) in the absence of the enzyme. d) Luminescence output of 0.1 μ M of protected D-thioluciferin thioacrylate (IX)(sulphide), D-thioluciferin homodisulphide (3')(disulphide), and free D-thioluciferin (3)(thiol) at a final luciferase concentration of 10 nM (Control readings were recorded for substrates in the absence of the luciferase enzyme). The Relative Light Units (RLUs) were determined in triplicate and are represented as the mean \pm SEM. e) Thiol-sensitive thioacrylate protected D-thioluciferin (IX) probe and redox reaction of D-thioluciferin (3).

Since it was recently reported that 6'-sulphides could be potential inhibitors for the WT luciferase enzyme, the thioacrylate-protected D-thioluciferin (IX) was further evaluated as an inhibitor of luciferase where it was shown to be strongly inhibitory (Figure S6). The sulphide's inhibition of luciferase could similarly be used to inform the design of D-thioluciferin (3)-based probes.

Kinetics

Using a plot of initial rates, the apparent K_m of D-thioluciferin (3) was calculated using the K_m 's for D-luciferin (1) and D-aminoluciferin (2) as references. The apparent K_m was then calculated as 0.09801 μ M, which was on the same order as that previously calculated for (2) (0.39-0.69 μ M) and related analogues (Figure S7), and consistent with the 0.16 μ M reported by Pirrung et al.^{15,21} The K_m was, surprisingly, much lower than that of the native substrate, (1) (8.3 μ M), despite the lower emission intensity at the same concentration (Table S8).²¹⁻²² The latter result, along with the fact that the substrate (3) combined with the luciferase light-emitting

reaction has a lower quantum yield compared to (1), may shed some light on the bioluminescence activity of (3).

Conclusions

The synthesis of D-thioluciferin and the S-protected-thioacrylate have now paved the way for the development of novel biothiol-relevant applications. The kinetics of D-thioluciferin release, in the case of S-protected-thioacrylate, is expected to be more favourable compared to thiophenol and needs to be evaluated further under physiological conditions. The lower K_m and longer, red shift of λ_{max} relative to D-luciferin and D-aminoluciferin make D-thioluciferin a promising bioluminogenic candidate whose properties and applications should be further explored. Moreover, thioluciferin provides a unique handle that readily allows for bioluminescence to be coupled to biologically relevant sulphur chemistry. By exploiting the difference in bioluminescent activity of thioluciferin and its oxidised forms, e.g., disulphide, one can envisage several potential applications which should be investigated further. The S-protected-thioacrylate can be utilized as a general thiol sensor. Furthermore, the established synthetic methodology for unsymmetrical disulfides would allow for the synthesis of disulfide reductase substrates that would release the bioluminescent D-thioluciferin molecule upon enzyme cleavage. This may then lead to the development of new bioluminescent sensors based on the D-thioluciferin molecule.

Experimental Section

General. All reactions were carried out in oven-dried glassware under an inert nitrogen atmosphere, unless otherwise stated. Reagents were obtained from commercial sources (Sigma–Aldrich, Merck) and used as received unless otherwise stated. Solvents were evaporated under reduced pressure at 40 °C using a Buchi Rotavapor, unless otherwise stated. Aqueous solutions were prepared using distilled water. All reactions were monitored by TLC using aluminum-backed Merck silica-gel 60 F254 plates, and compounds were visualised on TLC under a UV-lamp and/or sprayed with a 2.5% solution of *p*-anisaldehyde in a mixture of sulfuric acid and ethanol (1:10 v/v), iodine vapour or ceric ammonium sulphate solution, and then heated using a 1600 W heat gun. Normal-phase column chromatography was carried out using silica-gel (Fluka Silica Gel 60, 40-63 microns), and compounds eluted with the appropriate solvent mixtures. All compounds were dried under vacuum before yields were determined and spectroscopic analyses performed. Purity was determined by analytical chromatography using an Agilent HPLC 1260 equipped with an Agilent infinity diode array detector (DAD) 1260 UV-Vis detector, with an absorption wavelength range of 210 - 640 nm. The compounds were eluted using a mixture of 10 mM $\text{NH}_4\text{OAc}/\text{H}_2\text{O}$ and 10 mM $\text{NH}_4\text{OAc}/\text{MeOH}$ at a flow rate of 0.9 mL.min⁻¹ (10% $\text{NH}_4\text{OAc}/\text{MeOH}$ between 0 and 1 min, 10 - 95% $\text{NH}_4\text{OAc}/\text{MeOH}$ between 1 and 3 min, 95% $\text{NH}_4\text{OAc}/\text{MeOH}$ between 3 and 5 min).

Nuclear Magnetic Resonance (NMR) spectra (¹H and ¹³C) were recorded on either a Bruker XR400 MHz spectrometer (¹H at 400.0 MHz and ¹³C at 100.6 MHz), Varian Mercury XR400 MHz spectrometer (¹H at 400.0 MHz and ¹³C at 100.6 MHz) or a Varian Mercury XR300 MHz spectrometer (¹H at 300.1 MHz and ¹³C at 75 MHz), and were carried out in CDCl_3 , $\text{DMSO}-d_6$ and D_2O as the solvents, unless otherwise stated. Chemical

shifts (δ) and coupling values (J) were reported in ppm and Hz, respectively. Chemical shifts for ^1H and ^{13}C were recorded using tetramethylsilane (TMS) as the internal standard.

Melting points were obtained using a Reichert-Jung Thermovar hot-stage microscope (HSM) and are uncorrected. Optical rotations were obtained using a Perkin Elmer 141 polarimeter at 20 °C. The concentration c refers to g/100 mL.

LCMS analyses were carried out with a UHPLC Agilent 1290 Infinity Series (Germany), accurate mass spectrometer Agilent 6530 Quadrupole Time of Flight (QTOF) equipped with an Agilent jet stream ionisation source (positive ionization mode) (ESI+) and column (Eclipse + C18 RRHD 1.8 μm :2.1 X 50, Agilent, Germany).

4,5-Dichloro-1,2,3-dithiazol-1-ium chloride (Appel's Salt).²⁴ Sulfur monochloride (13 mL, 158 mmol, 5 eq) was added to a solution of chloroacetonitrile (2 mL, 31.6 mmol, 1 eq) in anhydrous DCM (15 mL) in an oven-dried flask equipped with a gas outlet. Thereafter, the reaction mixture was swirled for a few seconds and then left to stand, without further agitation, for 24 h at room temperature under a nitrogen atmosphere. The resulting brown precipitate that had formed was filtered under vacuum and washed copiously with DCM (3 x 200 mL) to afford Appel's salt as a brown-green solid (5.30 g, 80%). Mp 117-130 °C. MS (ESI+): m/z Calculated for $\text{C}_2\text{Cl}_3\text{NS}_2$ [M+H] 207.8616, found 207.8610.

Benzyl propiolate. Cesium carbonate (5.81 g, 17.9 mmol, 3 eq) was suspended in DMF (5 mL) and cooled to 0 °C. The reaction mixture was allowed to stir and propiolic acid (0.44 mL, 7.14 mmol, 1.2 eq) was added dropwise to the stirring cooled suspension. After the addition, the resulting solution was left to stir for a further 20 min, while being maintained at 0 °C. Benzyl bromide (0.71 mL, 5.95 mmol, 1 eq) was then slowly added and, thereafter, the reaction was allowed to warm to rt at which point it was left to stir for an additional 10 min. The reaction mixture was then diluted with diethyl ether (15 mL) and washed with a saturated brine solution (5 x 10 mL). The organic layer was dried over MgSO_4 and reduced *in vacuo* to afford benzyl propiolate as a yellow oil (0.940 g, 99% yield). ^1H -NMR (400 MHz, CDCl_3) δ 7.40 (1H, m, -Ph), 5.23 (2H, s), 4.60 (1H, s) ppm. ^{13}C -NMR (100.6 MHz, CDCl_3) δ 152.3, 135.34, 129.03, 129.0, 128.9, 79.8, 67.96 ppm.

Benzyl (Z)-3-((4-aminophenyl)thio)acrylate (Id). Benzyl propiolate (0.580 g, 3.63 mmol, 1 eq) and *p*-aminothiophenol (0.450 g, 3.63 mmol, 1 eq) were stirred in anhydrous DMF (2 mL) at 0 °C under a nitrogen atmosphere for 24 h. The resulting red-brown solution was diluted with ethyl acetate (12 mL), washed with brine (4 x 6 mL), dried over MgSO_4 , and excess solvent was reduced *in vacuo*. The crude residue (E/Z mixture, Z-major) was then subjected to column chromatography eluting with 3:10 ethyl acetate:petroleum ether, and the material obtained was recrystallised to a constant melting-point temperature from boiling petroleum ether to afford the geometrically pure benzyl (Z)-3-((4-aminophenyl)thio)acrylate (**IId**) as a brown solid (0.276 g, 39%). Mp 118-119 °C. ^1H -NMR (300 MHz, CDCl_3) δ 7.36 (5H, m), 7.32 (1H, d, J 10.1 Hz), 7.14 (2H, d, J 8.5 Hz), 6.60 (2H, d, J 8.5 Hz), 5.89 (1H, d, J 10.1 Hz), 5.47 (2H, br s, $-\text{NH}_2$), 5.13 (2H, s) ppm. ^{13}C -NMR (100.6 MHz, CDCl_3) δ 165.8, 154.6, 149.9, 136.8, 133.4, 128.9, 128.5, 128.4, 119.4, 115.0, 111.5, 65.6 ppm. MS (ESI+): m/z Calculated for $\text{C}_{16}\text{H}_{15}\text{NO}_2\text{S}$ [M+H] 286.0901, found 286.0889.

Benzyl (Z)-3-(((Z)-4-chloro-5H-1,2,3-dithiazol-5-ylidene)amino)phenyl)thio)acrylate (IId). Appel's salt (0.220 g, 1.08 mmol, 2 eq) and benzyl (Z)-3-((4-aminophenyl)thio)acrylate (**Id**) (0.150 g, 0.540 mmol, 1 eq) were dissolved in DMF (4 mL). The resulting solution was stirred at room temperature under a nitrogen atmosphere for 1 h. Thereafter, anhydrous pyridine (0.05 mL, 1.08 mmol, 2 eq) was slowly added to the solution, after which it was left to stir for an additional 2 h. The mixture was then concentrated *in vacuo* and purified by silica column chromatography, eluting with 1:9 ethyl acetate:petroleum ether, to afford the title compound as a bright yellow solid (0.225 g, 99%). Mp 130-152 °C. ^1H -NMR (300 MHz, DMSO) δ 7.66 (3H, m), 7.41 (5H, m), 7.28 (2H, d, J 8.5 Hz), 6.07 (1H, d, J 10.0 Hz), 5.20 (2H, s). ^{13}C -NMR (100.6 MHz, DMSO) δ 165.8,

160.6, 151.2, 150.3, 147.2, 136.6, 132.7, 132.3, 128.9, 128.6, 128.5, 121.1, 113.4, 65.9 ppm. MS (ESI+): m/z Calculated for $C_{18}H_{13}ClN_2O_2S_3$ [M] 420.9905, found 420.9899.

Benzyl (Z)-3-((4-((cyanocarbonothioyl)amino)phenyl)thio)acrylate (IIId). DBU (0.13 mL, 0.854 mmol, 3 eq) was added, dropwise over 30 min, to a solution of (Z)-3-((4-((Z)-4-chloro-5H-1,2,3-dithiazol-5-ylidene)amino)phenyl)thio)acrylate (**IIId**) (0.120 g, 0.285 mol, 1 eq) in DMSO (2 mL) at room temperature under a nitrogen atmosphere. The resulting red-brown mixture was stirred for 40 min, after which it was diluted with ethyl acetate (12 mL). The reaction mixture was then washed with a saturated NH_4Cl solution (3 x 6 mL) and H_2O (1 x 10 mL). The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by silica gel column chromatography, eluting with 2:8 ethyl acetate : hexane, to provide the title compound as a red-orange solid (0.0480 g, 61%). Mp 96-97 °C. 1H -NMR (400 MHz, DMSO) δ 13.56 (1H, br s, -NH), 7.98 (2H, d, J 8.9 Hz), 7.67 (3H, app dd, J 9.5, 7.7 Hz), 7.41 (5H, m), 6.10 (1H, d, J 10.0 Hz), 5.20 (2H, s). ^{13}C -NMR (100.6 MHz, DMSO) δ 165.8, 161.8, 149.2, 137.9, 136.6, 134.7, 130.9, 131.3, 128.6, 128.5, 123.8, 114.2, 113.9, 66.0. MS (ESI+): m/z Calculated for $C_{18}H_{14}N_2O_2S_2$ [M+H] 355.0574, found 355.0580.

Benzyl (Z)-3-((2-cyanobenzo[d]thiazol-6-yl)thio)acrylate (IVd). Palladium chloride (4.00 mg, 0.0200 mmol, 0.1 eq), copper iodide (19.0 mg, 0.0990 mmol, 0.5 eq), TBAB (0.127 g, 0.394 mmol, 2 eq) and (Z)-3-((4-((Z)-4-chloro-5H-1,2,3-dithiazol-5-ylidene)amino)-phenyl)thio)acrylate (**IIId**) (70.0 mg, 0.197 mmol, 1 eq) were suspended in DMSO (1 mL). The resultant orange-red mixture was placed under a nitrogen atmosphere and stirred at 120 °C for 4 h. The reaction mixture was then diluted with EtOAc (6 mL) and washed with a saturated brine solution (4 x 2 mL). The organic layer was then dried over Na_2SO_4 , filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography 3:7 ethyl acetate:hexane to provide the 2-cyanobenzothiazole (**IVd**) as a yellow solid (0.0430 g, 62%). Mp 113-120 °C. 1H -NMR (400 MHz, DMSO) δ 8.59 (1H, d, J 1.9 Hz), 8.29 (1H, d, J 8.7 Hz), 7.85 (1H, dd, J 8.7, 1.9 Hz), 7.78 (1H, d, J 10.0 Hz), 7.41 (5H, m), 6.17 (1H, d, J 10.0 Hz), 5.22 (2H, s). ^{13}C -NMR (100.6 MHz, DMSO) δ 165.9, 151.4, 148.6, 138.4, 137.0, 136.8, 136.5, 130.1, 128.9, 128.6, 128.6, 125.6, 124.3, 114.5, 113.7, 66.1 ppm. MS (ESI+): m/z Calculated for $C_{18}H_{12}N_2O_2S_2$ [M+H] 353.0418, found 353.0542.

(S,Z)-2-(6-((3-(benzyloxy)-3-oxoprop-1-en-1-yl)thio)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (IX). To a stirring solution of benzyl (Z)-3-((2-cyanobenzo[d]thiazol-6-yl)thio)acrylate (**IVd**) (40.0 mg, 0.110 mmol, 1 eq) in DMSO (4 mL) at room temperature under a nitrogen atmosphere was added D-cysteine (20.0 mg, 0.110 mmol, 1 eq) in H_2O (6 mL). The solution was left to stir for 5 min, cooled to 0 °C, and then potassium carbonate (150.0 mg, 0.110, 1 eq) was added. The reaction mixture was then left to stir a further 10 min after which the pH was adjusted to pH 3 using 3 M HCl, all whilst maintaining a reaction temperature of 0 °C. The solution was then allowed to warm to room temperature and diluted with ethyl acetate (10 mL) and washed with H_2O (4 x 10mL). The organic layer was then dried over $MgSO_4$, filtered and evaporated under reduced pressure at 35 °C to afford a red oil. The crude material was further purified using silica column chromatography, eluting with 1:9 methanol:dichloromethane to afford the title compound as a red oil (50.0 mg, 99%). 1H -NMR (300 MHz, DMSO) δ 8.47 (1H, d, J 1.9 Hz), 8.20 (1H, d, J 8.6 Hz), 7.76 (2H, m), 7.40 (5H, m), 6.13 (1H, d, J 10.1 Hz), 5.47 (1H, dd, J 9.7, 8.5 Hz), 5.22 (2H, s), 3.77 (2H, m). MS (ESI+): m/z Calculated for $C_{21}H_{16}N_2O_4S_3$ [M+H] 457.0350, found 457.0341.

(S)-2-(6-mercaptobenzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid or D-thioluciferin (3). D-Cysteine hydrochloride monohydrate (15.0 mg, 0.0790 mmol, 2.01 eq) and benzyl (Z)-3-((2-cyanobenzo[d]thiazol-6-yl)thio)acrylate (**IX**) (14.0 mg, 0.0397 mmol, 1 eq) were suspended in DMSO (0.4 mL) at room temperature under a nitrogen atmosphere. Potassium carbonate (35.0 mg, 0.119 mmol, 3 eq) dissolved in water (0.4 mL) was then added to the mixture, and the resulting bright orange solution was stirred under an inert atmosphere for a further 10 min. Upon consumption of benzyl (Z)-3-((2-cyanobenzo[d]thiazol-

6-yl)thio)acrylate (**IX**), as evidenced by TLC analysis, the methanol was removed *in vacuo* and the remaining aqueous solution acidified to pH 3 with 3 M HCl. The aqueous was then extracted with ethyl acetate (5 x 1 mL). The combined organics were dried over NaSO₄, filtered, concentrated *in vacuo* and purified with column chromatography (1:9:0.1 methanol:dichloromethane:trifluoroacetic acid solvent system) to provide D-thioluciferin **3** as a pale yellow solid (11.0 mg, 99%). [α]_D²⁰: -11° (DMF, c = 1). Mp 89-90 °C. ¹H-NMR (400 MHz, DMSO) δ 8.49 (1H, s), 8.18 (1H, dd, *J* 8.7, 0.5 Hz), 7.78 (1H, dd, *J* 8.7, 2.0 Hz), 5.45 (1H, dd, *J* 9.8, 8.3 Hz), 3.75 (2H, m) ppm. ¹³C-NMR (100.6 MHz, DMSO) δ 177.5, 166.3, 163.2, 155.4, 150.5, 130.0, 125.5, 123.0, 121.9, 82.1, 34.5 ppm. HRMS (ESI+): *m/z* Calculated for C₁₁H₈N₂O₂S₃ [M+Na] 318.9645, found 319.0000.

Bioluminescence emission spectra

Bioluminescence emission spectra with purified firefly luciferase (luciferase from *Photinus pyralis*, Sigma-Aldrich) were recorded on a Varian Cary Eclipse fluorometer equipped with a regulated temperature cell holder using Hellma, Suprasil® quartz fluorescence cuvettes of 10 mm path length and 1.5 mL volume capacity. Reactions were initiated at ambient temperature in 1-ml total reaction volumes by injecting equal volumes of 10 nM enzyme in enzyme buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 1 mM DTT, and 0.8 mg/mL BSA) into 100 μ M of substrate in substrate buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 8 mM MgSO₄, and 4 mM ATP). The emission spectra were recorded 10 s post-addition and in 1 nm increments from 200 nm to 800 nm with a 0.1 second integration time and closed excitation slits.

Purified luminescence assays

Luciferase was prepared as a 10 nM solution in enzyme buffer. Luciferase-lyophilized powder (luciferase from *Photinus Pyralis*, Sigma-Aldrich) was dissolved in previously prepared luciferase enzyme buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 1 mM DTT, and 0.8 mg/mL BSA). D-Luciferin (**1**), D-aminoluciferin (**2**) and D-thioluciferin (**3**) were prepared as 0.1 μ M, 3.56 μ M, 10 μ M, 100 μ M and 1 M solutions in substrate buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 8 mM MgSO₄, and 4 mM ATP). Luminescence assays were initiated by adding 30 μ L of luciferase in luciferase enzyme buffer to 60 μ L substrate in substrate buffer in a white 96-well plate (Costar 3915). Imaging was performed one minute after enzyme addition using a Luminoskan Ascent (detector 270 - 670 nm) and data acquisition and analyses were performed with Ascent Software version 2.6. Data are reported as RLU for each ROI corresponding to each well of the 96-well plate.

Kinetic reaction curves

Kinetic reaction curves with purified firefly luciferase (luciferase from *Photinus pyralis*, Sigma-Aldrich) were recorded at a 10 nM final enzyme concentration. Experiments were performed as outlined above for bioluminescence emission spectra with the following changes. Measurements were taken every 5 s post-addition for 20 min.

UV-vis experiments

Absorption spectra for D-Luciferin (**1**) and luciferin-analogues were recorded with a Cary 60 spectrophotometer, instrument version 2.00. Samples were prepared as a 0.01 mM DMSO solution and were scanned from 200 nm to 1000 nm at a UV-Vis scan rate of 24000.00 nm/min. Data were analysed using Scan Software Version 5.0.0.999.

Fluorescence spectrophotometry

Excitation and emission spectra were recorded on a Varian Cary Eclipse fluorometer equipped with a regulated temperature cell holder and Hellma, Suprasil® quartz fluorescence cuvettes of 10 mm pathlength and 1.5 mL volume capacity. Emission spectra were recorded at an excitation wavelength corresponding to previously recorded absorption maxima. Samples were prepared as a 0.1 mM DMSO solution and were scanned from 200 nm to 800 nm (Ex. Slit/ Em. Slit 5 nm) at a scan rate of 600.00 nm/min. Data were analysed using Scan Software Version 1.1.

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Supplementary Material

Supplementary material, consisting of excitation and emission spectra (Figure **S1-S5**), luminescence data for luciferins (Table **S8**), enzyme kinetics studies (Figure **S7**) and ^1H and ^{13}C NMR and Mass spectra can be found in the online version.

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