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Authors
Pirrung, Michael C
Carlson, Andrew D
De Howitt, Natalie
et al.

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Synthesis and Bioluminescence of Thioluciferin

Michael C. Pirrung[1,a,b,c], Andrew D. Carlson[a], Natalie De Howitt[a,b], and Jiayu Liao[b]

[a] *Department of Chemistry, University of California-Riverside, Riverside, CA 92521 USA*
[b] *Department of Bioengineering, University of California-Riverside, Riverside, CA 92521 USA*
[c] *Department of Pharmaceutical Sciences, University of California-Irvine, Irvine, CA 92697 USA*

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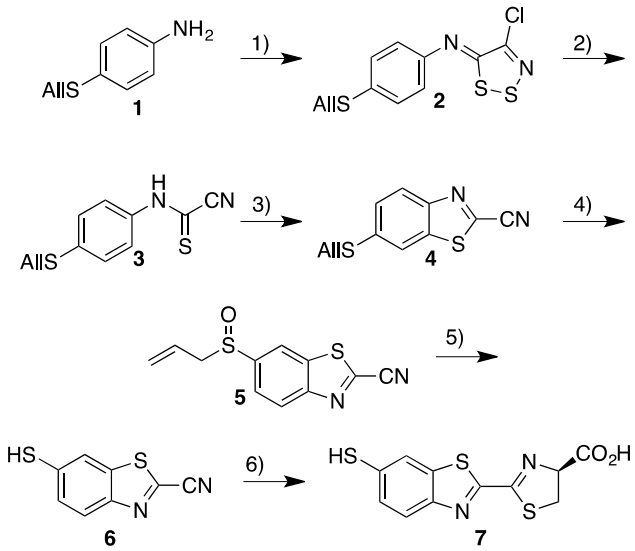
ABSTRACT

The firefly luciferin analog thioluciferin (S-luc) was synthesised as a key element of bioluminescent reporters for oxidation state and thiol/disulfide equilibria. It shows blue-shifts in absorption and fluorescence compared to luciferin, and is a modest luciferase substrate. These features are attributed to a π-system that is less conjugated than luciferin.

Thiols and their disulfide counterparts play widespread and crucial roles in biomolecular structure, function, and stability, commonly providing a redox buffering/sensing system and unique chemical reactivity. Many techniques to chemically assay biological thiols have been developed. Yet, methods to monitor changes in redox conditions in living cells continue to be challenging.[1] The broad utility of the bioluminescent reaction of D-luciferin (luc) with firefly luciferases in bioassays of reporters / labels has surprisingly not been extended to thiols/disulfide equilibria. We envisioned a novel bioluminescent method to report thiol/disulfide status (often dependent on mitochondrial activity) based on luciferase that offers the additional attraction of amenability to imaging. This concept requires a thiol analog of the luciferase substrate luciferin, which is heretofore unknown. Significant structural perturbations to the luciferin chromophore had not been made until recent years.[2] Here, a known luciferin synthesis was applied to the preparation of thioluciferin (S-luc) and its bioluminescent properties were investigated with firefly luciferase. Thioluciferin could provide a means to detect any oxidizing agent that forms disulfides, including nitric oxide and its decomposition products.

The synthesis of S-luc begins with (allylthio)aniline, which can be subjected to the general luciferin synthesis developed by Prescher,[3] involving Appel's dithiazolium salt.[4] The resulting **2** was fragmented to the cyanothioanilide **3**, followed by palladium-catalyzed ortho C-H activation to construct benzothiazole **4**. Prescher subsequently reported that direct conversion of dithiazoles like **2** to cyanobenzothiazoles can be accomplished simply by pyrolysis, after we had achieved that reaction catalytically.

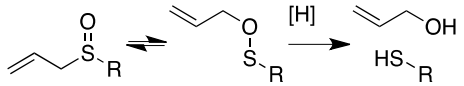


Scheme 1. 1) Appel's salt, pyr, 77%; 2) DBU, 83%; 3) cat. $PdCl_2$, CuI, TBAB, Δ, 65%; 4) $NaIO_4$, 90%; 5) $(PhO)_3P$, Δ, 79%; 6) D-cys, 87%.

A variety of literature methods were examined without success to remove the *S*-allyl protecting group from **4** to set the stage for luciferin formation. We therefore developed an alternative based on the well-known Mislow-Evans rearrangement.[5] It is typically used for the preparation of an

[1] Corresponding author. phone: +1-951-827-2722; e-mail: michael.pirrung@ucr.edu

allylic alcohol target, starting from an allylic sulfoxide that rearranges to an intermediate allylic sulfenate that is cleaved by a reductant (Scheme 2). In principle, it could also be used to release the thiol component of the sulfenate. To test this idea with **4**, sulfoxide **5** was obtained by periodate oxidation. Upon heating with triphenylphosphite, the desired thiol was produced. It is important to use triphenylphosphite in this process, as trimethylphosphite is converted to trimethylphosphate that methylates the released thiol. A very similar method has been reported for allylic selenide deprotection.[6] The final step in the preparation of S-luc (**7**, thioluciferin) from **6** simply uses the conventional luciferin synthesis with D-cysteine. The final product was fully characterized physicochemically and spectrally.



Scheme 2.

The UV-vis spectrum of S-luc is shown in Fig. 1. A notable feature is the ca. 6-fold hypochromicity of the main absorption band compared to luc (ε 3.2 $mM^{-1}•cm^{-1}$ vs. 18.2 $mM^{-1}•cm^{-1}$ for luciferin). The λ_{max} is also blue-shifted; likewise the weaker band at shorter wavelength. For comparison, thiophenol exhibits a 34 nm blue-shift compared to phenol. These observations likely reflect reduced conjugation of the thiol with the aromatic system compared to the phenol of luc, as might be expected from the larger 3p orbitals of sulfur. This implication is strengthened by optical measurements discussed below. We analyzed our initial samples of S-luc by RP HPLC (10% ACN in water) and saw fast- and slow-eluting peaks; the latter, the disulfide, could be eliminated by addition of the water-soluble phosphine TCEP.[7] Diode-array detection allowed the absorption spectra (Fig. S1) of both forms to be obtained, with λ_{max} for the thiol at 305 nm and λ_{max} for the disulfide at 350 nm. TCEP affects only the end-absorption and was used to maintain the reduced form of S-luc in further studies. With no precaution to include redox partners, S-luc exists as a mixture of reduced and oxidized forms. We expected the disulfide would not be a luciferase substrate, as observed in the studies below. We considered determining the specific redox potential of this thiol/disulfide couple, which at least must be between that of O_2 and TCEP. However, we judged that point moot until a luciferase could be engineered to catalyze S-luc at levels approaching the native luciferin substrate. It is also irrelevant to the redox of S-luc disulfides with other thiols that can form in vivo.

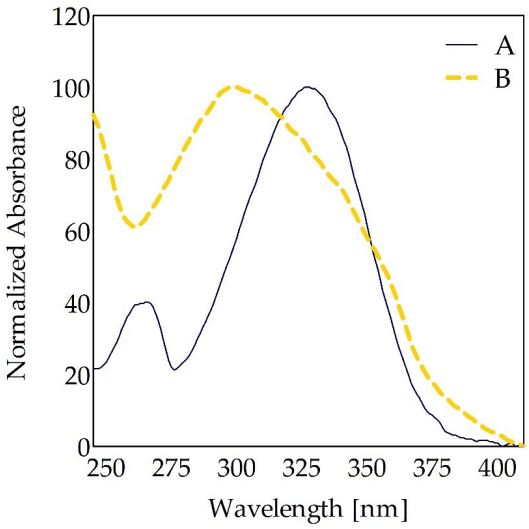


Figure 1. UV spectra of thioluciferin in unbuffered water compared to luciferin. A) luc, 0.16 mM; B) S-luc, 1 mM, 0.5 mM TCEP

Fluorescence emission spectra of luciferins can be useful in understanding the excited state properties of their derived oxyluciferins and their bioluminescence.[8] The fluorescence spectrum of S-luc was therefore determined. It shows trends similar to its UV spectrum - the emission is blue-shifted by about 30 nm (Fig. 2) and reduced in intensity by about 3-fold (Fig. S2) compared to luc. Omitting TCEP gives greater S-luc fluorescence owing to the contribution of the more fluorescent disulfide.

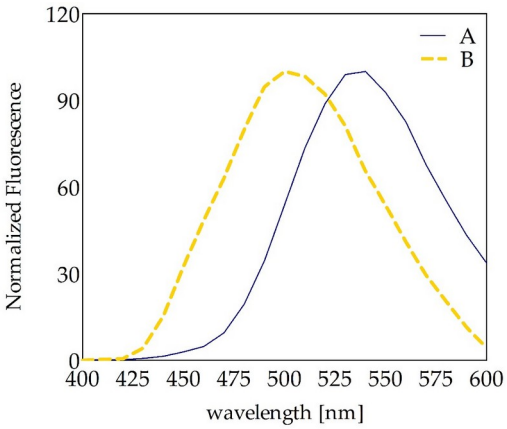


Figure 2. Normalized fluorescence emission spectra of luciferin (A, 10 μM) and thioluciferin (B, 10 μM). Excitation was at 328 nm for luc and 300 nm for S-luc.

With these optical properties as preamble, bioluminescence was examined with **7** as substrate for *Photinus pyralis* luciferase. These studies were performed in the presence of 1 mM TCEP, which control experiments show does not affect luciferase activity at that concentration; it does affect it, slightly, at 5 mM. No bioluminescence is seen in the absence of TCEP. The pH dependence of total bioluminescence is shown (Fig. 3). The maximum, at pH 9.4, is more basic than for luc, which is at pH 7.8.

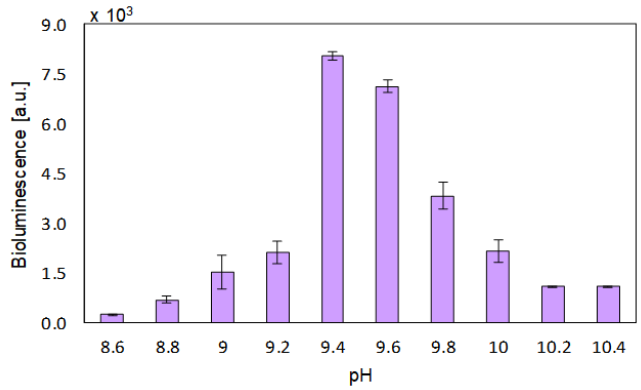


Figure 3. Total bioluminescence of *P. pyralis* luciferase with S-luc as substrate: pH dependence. Manual injection of luciferase to a final concentration of 1 μM was made into a reaction mix containing S-luc (1 μM final concentration), ATP, $MgSO_4$, TCEP and glycine-NaOH buffer. Assays were performed in triplicate and error bars represent standard deviations within the triplicate. Data were obtained using the instrument described in the experimental section.

Steady-state kinetics studies showed an abnormal saturation plot (Fig. S3), with reduced bioluminescence above 0.5 μM. A K_m of 0.16 μM was estimated from these data. Considering this behavior possibly due to substrate inhibition, we examined the ability of S-luc to inhibit the luciferase reaction with luc as the substrate. This experiment is straightforward to perform because S-luc has far less substrate activity than luc (vide infra). Analysis showed S-luc is a competitive inhibitor (Fig. S4) with a K_i of 0.07 μM. This study demonstrates that the low substrate activity of S-luc is not caused by diminished enzyme active site binding, consistent with the comparable K_m and K_i values.

The substrate activity of S-luc was compared with that of luc (each at its optimum pH). Because bioluminescence with **7** is far lower than luc, this required dilution of luciferase by 1000 to give comparable emissions. The efficiency of bioluminescence production from **7** was 5.4×10^4 less than with luc. The bioluminescence intensity with **7** was insufficient to obtain an emission spectrum using our equipment. High emission intensity has generally *not* been observed with other luciferin analogs, a phenomenon that is not well-understood. The one exception requires enzyme engineering.[9] The magnitude of the drop-off for S-luc was one of the larger seen, however.

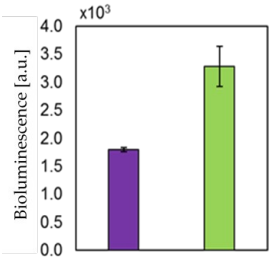


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Figure 4. Total bioluminescence of *P. pyralis* luciferase with S-luc (■) and luc (■) as substrate. The conditions for luc were its optimum pH and K_m, 7.8 and 1 μM, and for S-luc were its optimum pH, 9.4, and 0.5 μM. TCEP (1.5 μM was used to maintain it in the reduced form). The concentrations of luciferase used were 1 nM and 1 μM. Value represents the mean of triplicates and the error bars indicate standard deviation.

Two related sulfur-based luciferin analogs, 6′-methylthio-luciferin and 6′-methylsulfinyl-luciferin, have been reported by Miller.[10] The absorbance of the former is slightly red-shifted compared to luciferin, opposite to the trend observed here with S-luc. Its fluorescence is blue-shifted ca. 40 nm and there was a diminution of fluorescence quantum yield. The absorbance of the latter is blue-shifted 30 nm compared to luciferin, and it is not fluorescent. Neither of these compounds is a luciferase substrate.

The reduced conjugation of the thiol implied in the optical properties of **7** gives an explanation for its diminished bioluminescence. That is, owing to poorer p-orbital overlap of the thiolate with the aromatic system, S-luc is less similar to luc than it is to deoxyluciferin, which is not bioluminescent. A difference in emission depending on the electron-donating properties of the 6-substituent is the basis of many luminogenic enzyme assays that use derivatized aminoluciferins or luciferins. Commensurate absorption of deoxyluciferin is at 247 nm and 295 nm, compared to 269 nm and 330 nm for luciferin and 330 nm for S-luc.[12] These are blue-shifts of 22 nm and 35 nm.

The greater binding affinity of S-luc for the enzyme than the native substrate may relate to the greater hydrophobicity of S-luc, which has a Clog*P* of 3.11 compared to the Clog*P* of luc of 2.53. More attractive interactions with luciferase residues of the thiol than luciferin's phenol could also be involved.

There is a tension in the design of this system between potential oxidation of the S-luc thiol to the disulfide or other non-bioluminescent products and the requirement for oxygen in the conversion of any luciferin to the oxyluciferin excited state by luciferase. However, it is known that in thiol-disulfide interchange reactions, K_{eq} is mostly controlled by the pK_as of the thiols.[13] While the pK_a of S-luc was not determined, it was calculated (MarvinSketch 14.7.28, 2014) to be 5.6, far lower than most biological thiols. Barring highly oxidizing conditions, it should be possible to maintain **7** in the thiol form and design precursors to it by thiol-disulfide exchange. In some instances, the optimum pH for a particular luciferin analog has been found to be near its pK_a

While there are other publications using luciferase to report on the presence of thiols, they are complex and indirect.[14] The development of a luciferase-based reporter of cell redox status is still needed.

Keywords: firefly luciferase; luminescence; substituent effect; thiol; disulfide

References and notes

1. Winther, J. R.; Thorpe, C. *Biochim. Biophys. Acta – Gen. Subj*. **2014**, *1840*, 838-846. Leichert, L. I.; Jakob, U. *Antioxid. Redox Signal*. **2006**, *8*, 763-72. Gilbert, H. F. *Methods Enzymol*. **1995**, *251*, 8-28. Go, Y. M.; Jones, D. P. *Crit. Rev. Biochem. Mol. Biol*. **2013**, *48*, :73-81.
2. (a) Takakura, H.; Kojima, R.; Kamiya, M.; Kobayashi, E.; Komatsu, T.; Ueno, T.; Terai, T.; Hanaoka, K.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc*. **2015**, *137*, 4010-3.; (b) Reddy, G. R.; Thompson, W. C.; Miller, S. C. *J. Am. Chem. Soc*. **2010**, *132*,

13586; (c) Conley, N. R.; Dragulescu-Andrasi, A.; Rao, J.; Moerner, W. E. *Angew. Chem. Int. Ed*. **2012**, *51*, 3350; (d) Sun, Y. Q.; Liu, J.; Wang, P.; Zhang, J.; Guo, W. *Angew. Chem. Int. Ed*. **2012**, *51*, 8428; (e) Takakura, H.; Kojima, R.; Ozawa, T.; Nagano, T.; Urano, Y. *ChemBioChem* **2012**, *13*, 1424; (f) Woodroofe, C. C.; Meisenheimer, P. L.; Klaubert, D. H.; Kovic, Y.; Rosenberg, J. C.; Behney, C. E.; Southworth, T. L.; Branchini, B. R. *Biochemistry* **2012**, *51*, 9807; (g) Takakura, H.; Sasakura, K.; Ueno, T.; Urano, Y.; Terai, T.; Hanaoka, K.; Tsuboi, T.; Nagano, T. *Chem. Asian J*. **2010**, *5*, 2053.

3. McCutcheon, D. C.; Paley, M. A.; Steinhardt, R. C.; Prescher, J. A. *J*. Am. Chem. Soc. **2012**, *134*, 7604. McCutcheon, D. C.; Porterfield, W. B.; Prescher, J. A. *Org. Biomol. Chem*. **2015**, *13*, 2117-21.
4. Appel, R.; Janssen, H.; Siray, M.; Knoch, F. *Chem. Ber.* **1985,** *118*, 1632 – 1643. Cuadro, A. M.; Alvarez-Buila, J. *Tetrahedron*, **1994**, *50*, 10037-10046. English, R. F.; Rakitin, O. A.; Rees, C. W.; Vlasova, O. G. *J. Chem. Soc., Perkin Trans. 1* **1997**, *3*, 201 – 206.
5. Evans, D. A.; Andrews, G. C. *Acc. Chem. Res.* **1974**, *7*, 147–155.
6. Abbas, M.; Wessjohann, L. A. *Org. Biomol. Chem*. **2012**, *10*, 9330-9333.
7. Getz, E. B.; Xiao, M.; Chakrabarty, T.; Cooke, R.; Selvin, P. R. *Anal. Biochem*. **1999**, *273*, 73–80.
8. White, E. H.; Roswell, D. F. *Photochem. Photobiol*. **1991**, *53*, 131-6. Navizet, I.; Roca-Sanjuán, D.; Yue, L.; Liu, YJ.; Ferré, N.; Lindh, R. *Photochem. Photobiol*. **2013**, *89*, 319-25.
9. Mofford, D. M.; Reddy, G. R.; Miller, S. C. *J. Am. Chem. Soc*. **2014**, *136*, 13277-82.
10. Sharma, D. K.; Adams, Jr., S. T.; Liebmann, K. L.; Miller, S. C. *Org. Lett*. **2017**, *19*, 5836-5839.
11. Denburg, J. L.; Lee, R. T.; McElroy, W. D. *Arch. Biochem. Biophys*. **1969**, *134*, 381-394.
12. White, E. H.; Wörther, H.; Field, G. F.; McElroy, W. D. *J. Org. Chem*. **1965**, *30*, 2344-2348.
13. Singh, R.; Whiteside, G. M. in *The chemistry of sulphur containing functional groups*, Patai, S.; Rappoport, Z., Eds., John Wiley & Sons, **1993**, 633-658.
14. Burgos, E. S.; Gulab, S. A.; Cassera, M. B.; Schramm, V. L. *Anal. Chem*. **2012**, *84*, 3593-3598. Hemmi, M.; Ikeda, Y.; Shindo, Y.; Nakajima, T.; Nishiyama, S.; Oka, K.; Sato, M.; Hiruta, Y.; Citterio, D.; Suzuki, K. *Chem. Asian J*. **2018**, *13*, 648.

Supplementary Material

Supporting information for this article totaling 26 pages, including experimental procedures for the synthesis of S-luc and its assay as well as NMR spectra and other figures, can be obtained from the corresponding author or can be found in the online version, at http://dx.doi.org/10.1016/xxxx.