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Coumarin luciferins and mutant luciferases for robust multicomponent bioluminescence imaging

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ABSTRACT: Multicomponent bioluminescence imaging requires an expanded collection luciferase-luciferin pairs that emit far-red or near-infrared light. Toward this end, we prepared a new class of luciferins based on a red-shifted coumarin scaffold. These probes (CouLuc-1s) were accessed in a two-step sequence via direct modification of commercial dyes. The bioluminescent properties of the CouLuc-1 analogs were also characterized, and complementary luciferase enzymes were identified using a two-pronged screening strategy. The optimized enzyme-substrate pairs displayed robust photon outputs and emitted a significant portion of near-infrared light. The CouLuc-1 scaffolds are also structurally distinct from existing probes, enabling rapid multicomponent imaging. Collectively, this work provides novel bioluminescent tools along with a blueprint for crafting additional probes for multiplexed imaging.

INTRODUCTION

Bioluminescent enzymes (luciferases) are among the most popular reporters for imaging biological processes in vitro, in live cells, and in animal models.1-2 Luciferases generate light via the oxidation of small molecule luciferins (Figure 1a). Since no external light source is needed, bioluminescent probes offer high signal-to-noise ratios in heterogeneous environments.2,3 The remarkable sensitivity, combined with the broad dynamic range, has made bioluminescence a go-to imaging technique for tracking cell movements, proliferation, and numerous other features in living organisms.4-8

While powerful, bioluminescence has been slow to transition to imaging multiple targets simultaneously, owing to a lack of distinguishable probes.9 Multiplexed imaging is possible using spectrally resolved luciferase-luciferin pairs.10-13 Indeed, several luciferin analogs have been developed that emit different colors of light.14-16 The emission profiles can be further tuned with engineered luciferases17-18 or luciferase-fluorescent probe fusions.19,20 In many cases, though, the spectral resolution achieved is insufficient for routine application in vivo. Sensitive imaging in whole organisms requires >650 nm light, as these wavelengths are significantly less absorbed by tissue.21 However, few bioluminescent probes emit light in the requisite far-red to near-infrared (NIR) range. The perceived color of emission in traditional bioluminescent detection also changes with the depth of the source, complicating the assignment of different colored probes.24

Figure 1. Red-emitting orthogonal bioluminescent probes designed from fluorophores. (a) D-Luciferin is oxidized by firefly luciferase (Fluc) to produce oxyluciferin and a photon of light. (b) Coumarin fluorophores were used as templates for red-shifted luciferins. (c) Retrosynthetic analysis of the CouLuc-1 analogs.
Multiplexed bioluminescence imaging can also be achieved via substrate resolution—using luciferases that recognize different luciferin structures (i.e., orthogonal pairs). Light is produced when complementary enzymes and substrates react, but minimized in all other cases. A handful of such orthogonal probes have been co-opted for dual imaging in vivo, but applications have been mostly limited to monitoring superficial targets. This is due, in part, to a shortage of luciferins with both sufficient red emission and distinct molecular architectures. Candidate luciferins must also be sufficiently bright, bioavailable, and easy to synthesize—criteria that few existing probes meet. Consequently, routine multicomponent imaging with three or more red-emitting bioluminescent reporters remains challenging.

We surmised that unique classes of NIR-emitting luciferins could be developed using fluorescent scaffolds as guides (Figure 1b). Our approach was inspired by previous reports of luciferin analogs comprising entirely new heterocycles, including benzothiophene, quinoline, and coumarin derivatives. We also took cues from recent efforts to develop red-shifted luciferins by extending the π-conjugation of the scaffold and restricting conformational flexibility (e.g., AkaLumine and infraluciferin). In most cases, the engineered luciferins were found to be poor substrates for Fluc. Light emission could be recouped, though, via extensive enzyme engineering (e.g., generating Akaluc and CBR mutants).

We focused on a new class of luciferins (CouLuc-1s) comprising both an elongated π-system and a 4-trifluoromethylcoumarin (Figure 1c). The coumarin fluorophore is a well-established imaging agent and has been incorporated into other motifs to achieve NIR emission. The small size of the coumarin core would also likely require only minimal enzyme engineering to identify complementary luciferases. Given the unique structure of the CouLuc-1s, we further anticipated that the analogs could be used for multicomponent imaging with other red-emitting probes, including AkaLumine/Akaluc and furimazine/ANTares.

Here we detail the synthesis and evaluation of the CouLuc-1 probes. We developed a two-step route to bridge the fluorescent coumarin heterocycle with the key thiazone unit necessary for luciferin bioluminescence. The resulting conjugates displayed red-shifted emission. Complementary luciferases were identified via a parallel engineering approach. The resulting luciferase-luciferin pairs provided robust light outputs that were suitable for multiplexed imaging. Overall, these efforts provide a new class of easily accessible, long-wavelength bioluminescent pairs with significant promise for orthogonal imaging in vivo.

RESULTS AND DISCUSSION

Design and Synthesis of Coumarin-linked Luciferins. We set out to prepare CouLuc-1 analogs bearing different electron donors (–NMe2, –NH2, and OH) at C7 of the coumarin heterocycle. These modifications are commonly found in coumarin fluorophores and are known to modulate the excited state properties. Retrosynthetic analysis of the CouLuc-1 analogs revealed the key disconnection in the alkene linkage between the coumarin and thiazone (Figure 1c). We envisioned that if the olefin bridge could be installed with a nitride handle, subsequent cysteine condensation would afford the desired luciferins in a highly concise two-step sequence. While methods to directly modify the carbonyl group on coumarins are rare, recent reports suggested that thiolactones are viable intermediates for synthesizing π-extended coumarins. Additionally, direct thiazoline formation from non-activated nitriles can be difficult, as similar transformations typically proceed through a multi-step sequence from corresponding carboxylates.

Scheme 1. Synthesis of coumarin luciferin scaffolds.

Reaction conditions: (1) 1 (1.0 equiv), CH3CN (4.0 equiv), n-BuLi (4.0 equiv), THF, −78 °C, 15 min, then 0.5 M HCl, rt, 1-4 h; (2) 2 (1.0 equiv), d-Cys (1.5 equiv), NaHCO3 (4.0 equiv), EtOH, 85 °C, 80-120 h

With these synthetic challenges in mind, we set out to develop olefination conditions for installing a cyanomethylene moiety onto commercial coumarin starting materials. After examining several strategies, we found that cyanomethyl anions generated in situ from acetoniitride and n-BuLi react readily with coumarins (Scheme 1). Exposing 2a-c to d-cysteine and NaHCO3 in ethanol generated CouLuc-1 analogs 3a-c in 3-5 days. Following cycloaddition, a single isomer was formed, and the geometry was verified by 2D NOSEY (Figure S1). Overall, the route provided access to 3a-c in 26-32% yield in just two steps. This approach is among the shortest luciferin syntheses to date from readily available starting materials. To highlight the scalability of the route, we also developed a chromatography-free procedure to access 3a (Figures S2-S3).

In Vitro Characterization with Native Luciferase. With the CouLuc-1 analogs in hand, we first examined their bioluminescent properties. All three analogs produced light when combined with Fluc and the necessary cofactors (Figure 2a). Scaffolds with amino (CouLuc-1-NH2) or dimethylamino (CouLuc-1-NMe2) substituents exhibited stronger photon outputs than the hydroxy variant (CouLuc-1-OH, Figure 2b). Similar boosts in brightness have been achieved with other amino luciferins. Compared to the native Fluc substrate (d-luc), though, the CouLuc-1 derivatives produced lower levels of light (~1000-fold dimmer, Figure S4). The reduced brightness was partly attributed to low binding affinities between Fluc and the coumarin analogs (Figure S5). Similar trends have been observed with other sterically modified luciferins exhibiting comparable emission levels (Figure S6). While low, the photon outputs achieved with Fluc and the CouLuc-1 derivatives provided a clear starting point for evolving brighter luciferases.
In terms of color, CouLuc-1-NMe₂ and -OH exhibited peak emission wavelengths (λ_{max}) at 620 and 625 nm, respectively. CouLuc-1-NH₂ was slightly blue-shifted, with λ_{max} = 597 nm. Compared to d-luc, all analogs were red-shifted by 30-60 nm at 25 °C (Figure 2c). Nearly 40% of the emitted photons from CouLuc-1-NMe₂ and CouLuc-1-OH were >650 nm. For comparison, Fluc/d-luc emits only 5% of photons >650 nm in vitro (8% in cellulo).

We further compared the CouLuc-1 bioluminescent spectra to the corresponding fluorescence spectra. In aqueous media, the fluorescence readouts exhibited similar trends, with the observed maxima red-shifted by 70-100 nm compared to d-luc (Table S1). This strong correlation between CouLuc-1 fluorescence and bioluminescence is consistent with other pi-extended and amino luciferins. In CouLuc-1 fluorescence was also strongly influenced by solvent and pH (Figure S7, Table S2). In polar aprotic solvents, both amino CouLuc-1s were more blue-shifted (up to 170 nm) than in protic or aqueous environments. Two peaks were observed in the emission spectrum for CouLuc-1-OH at acidic pH (517 and 600 nm), likely due to different protonation states. Environmentally sensitive emission is common among coumarin derivatives.

Such solvatochromic behavior is attractive for bioluminescence imaging, as the “environment” can be tuned via engineering of the luciferase active site.

**Engineering complementary luciferases for CouLuc-1s.**

After establishing the CouLuc-1s as viable luminophores, we set out to improve photon outputs by reengineering the luciferase enzyme (Figure 3a). The ideal mutants would exhibit improved turnover, maintain red-shifted emission, and be selective for the coumarin scaffolds. We initially focused on CouLuc-1-NMe₂ due to its robust activity with Fluc and desirable spectral properties. We took a two-pronged screening approach to identifying mutants from library screens using (1) *in silico* design and (2) a semi-rational strategy. Both methods have been used to generate complementary enzymes for synthetic luciferins. In the first approach, we sculpted the luciferase active site using RosettaDesign. This strategy is useful for identifying mutations unique to an analog without significant prior knowledge or evolutionary starting point. To computationally identify mutations suited for the CouLuc-1 scaffold, we used the Rosetta-Match algorithm to dock CouLuc-1-NMe₂ with existing Fluc crystal structures. The model oriented the coumarin heterocycle toward the pocket normally adjacent to C4' on d-luc. In this configuration, the dimethylamino substituent was predicted to clash with the backbone of some active site residues (Figure S8), likely resulting in diminished turnover. We next employed RosettaDesign to resolve this clash and optimize the packing interaction between the coumarin luciferin and surrounding residues. From this analysis, a total of 41 sites were mutated to create a complementary active site for CouLuc-1-NMe₂ (Figure 3b).
ure S9). These residues were then ranked for targeting based on their proximity and known biochemical data. Twenty sites were ultimately selected for randomization via combinatorial codon mutagenesis (Figure S9).

The resulting Rosetta-inspired library was introduced into bacteria, and the transformed colonies were sprayed with CouLuc-1-NMe$_2$. Out of ~7,000 colonies screened on plate, ~150 were light-emitting (Figure S10). These colonies were collected, and the mutants were verified in two secondary screens (Figure S10). Variants with >10-fold improved photon output over Fluc were carried forward. Unique sequences were then validated in a second bacterial cell assay. From this workflow, two hits were identified (Figure 3b). Intriguingly, both variants contained a S347G mutation. We also screened the Rosetta-based library with CouLuc-1-OH. In this case, three hits were identified, with the point mutant S347G providing the most light (Figure S11). While only a subset of the Rosetta library was screened, the frequency of the S347G mutation among the ‘hits’ suggested that this residue is beneficial for CouLuc-1 processing. S347G is also known to stabilize the open conformation of the luciferase active site\(^{70-71}\) and process luciferins with steric bulk at C4'.

In parallel with the Rosetta approach, we screened CouLuc-1-NMe$_2$ against a focused library of 222 previously characterized Fluc mutants.\(^{29}\) The luciferases comprise mutations confined to the luciferin binding pocket and exhibit unique preferences for sterically and electronically modified luciferins.\(^{26,29}\) Screens of this library could provide additional information on residues underlying substrate specificity for the CouLuc-1 scaffolds. All three analogs were subjected to the focused library. Eleven hits were identified for CouLuc-1-NMe$_2$ (Figure 3c). A similar number of mutants were found to exhibit enhanced light emission for the -NH$_2$ and -OH analogs (Figure S12). The brightest mutants from these screens also comprised the S347G mutation, reinforcing the notion that residue 347 plays a pivotal role in CouLuc-1 processing. Another mutation common to multiple hits was F243M, a residue previously shown to aid in processing bulky luciferin analogs.\(^{29,56}\)

In Vitro and In Cellulo Characterization of Lead Mutant. From the screening hits, we selected the F243M/S347G mutant (dubbed Pecan) for additional characterization with CouLuc-1-NMe$_2$. Pecan was particularly attractive for orthogonal probe development as it has been previously used in vi-

![Figure 4. Photon output of CouLuc-1-NMe$_2$ with an engineered luciferase.](image)

(a) Kinetic studies revealed that Pecan could more efficiently process CouLuc-1-NMe$_2$ compared to Fluc. (b) Improved photon outputs were observed in cellulo. Pecan- or Fluc-expressing cells were incubated with either CouLuc-1-NMe$_2$ (250 μM) or D-luc (250 μM). Transfection efficiencies were determined via co-expression of GFP. Peak emission intensities were plotted as photon flux per cell. Error bars represent the standard error of the mean for n = 3 experiments. (c) In cellulo emission spectrum of Pecan/CouLuc-1-NMe$_2$ compared to other bioluminescent probes at 25 °C.

When CouLuc-1-NMe$_2$ was incubated with purified Pecan, more intense light emission (77-fold) was observed compared to Fluc (Figure S13). The boost in light output was likely due to the enhanced binding affinity of CouLuc-1s with Pecan, as revealed by kinetic analyses (Figure 4a and S14). Importantly, robust emission in the NIR region was maintained. Approximately 30% of photons produced by CouLuc-1-NMe$_2$ were >650 nm, and the emission spectra for the other analogs were similarly red-shifted (Figure S15).

After examining Pecan/CouLuc-1-NMe$_2$ in vitro, we evaluated the pair in mammalian cells. Pecan and Fluc were transiently expressed in HEK293 cells. The cells were incubated with either the coumarin analogs or n-luc. Peak photon outputs for each enzyme-substrate combination were measured and normalized to a common transfection marker (GFP). As shown in Figures 4b and S16, Pecan-expressing cells treated with CouLuc-1-NMe$_2$ emitted 14-fold more photons than Fluc-expressing cells. Similar improvements were observed when Pecan-expressing cells were incubated with either CouLuc-1-NH$_2$ or CouLuc-1-OH (Figure S17). The robust emission from CouLuc-1 analogs was also recapitulated in Pecan-expressing DB7 cells (Figure S18). We next compared the emission from Pecan/CouLuc-1-NMe$_2$ to both native and engineered bioluminescent pairs (e.g. Fluc/CyLuc1 and Aka-luc/AkaLumine). In both cases, the photon output compared favorably to the well-established bioluminescent pairs (Figure S19). The Pecan/CouLuc-1-NMe$_2$ pair also provided significant numbers of NIR photons (32% of photons >650 nm) in cells (Figure 4c). This level of NIR emission is on par with other state-of-the-art bioluminescent tools (Figure S20).

Multicellular imaging with Pecan and CouLuc-1-NMe$_2$. The optical properties of the CouLuc-1 analogs coupled with their unique structures made them promising candidates for multiplexed imaging. As noted earlier, resolving luciferases by substrate requires unique luciferin architectures. The more structurally diverse the luciferins, the better they can be distinguished by engineered enzymes.\(^{29}\) Substrate unmixing and image processing algorithms can rapidly detect unique enzyme-substrate pairings within complex mixtures.\(^{30,72}\) Multiple classes of orthogonal probes are available for multicomponent imaging via these methods, but only a few exhibit the necessary optical properties (NIR bioluminescence) for sensitive imaging.
Given the unique structures and robust emission of Pecan/CouLuc-1-NMe₂, we reasoned that this pair would be useful for multiplexing with other red-shifted probes. We were drawn to Akaluc/AkaLumine⁵⁵ and Antares/furimazine⁴⁷ as these pairs have been recently used to monitor tumor-immune interactions in vivo.¹¹ Pecan and Akaluc derive from the insect luciferase family and are thus inherently orthogonal to Antares, which uses a different mechanism for light emission. The CouLuc-1 scaffold is also structurally distinct from AkaLumine, suggesting Pecan and Akaluc could be readily differentiated based on their substrate preference. Indeed, minimal crosstalk was observed when Pecan was treated with AkaLumine or when the CouLuc-1 luciferin was added to Akaluc (Figure S21). The high level of orthogonality highlights the unique chemical space occupied by the coumarin analogs.

To test the multiplexed strategy, we plated Pecan-, Akaluc-, and Antares-expressing DB7 cells in varying ratios. Cell cultures containing a single reporter were also plated as controls (Figure 5a). Each substrate was added sequentially, and an image was acquired after each administration. The resulting images were analyzed using a linear unmixing algorithm,⁴⁰,⁷⁴ and the relative abundance of each cell type was false colored in a composite image (Figure 5a). Each luciferase-expressing cell was readily discerned, and the unmixed signals correlated with the number of cells present (Figure 5b). The entire triple component imaging was completed within 30 min, a notable improvement over traditional methods that require substrate clearance (multiple days). Altogether, these results suggest that Pecan/CouLuc-1-NMe₂ can be readily integrated with other engineered probes for rapid, multicellular imaging.

**Conclusion**

Despite the growing number of luciferin analogs, only a few are both chemically distinct and red-shifted. We have developed and characterized a new panel of structurally unique and near infrared-emitting luciferins based on a modified coumarin scaffold. The substrates were synthesized in just 2 steps without chromatographic purification. The CouLuc-1 analogs were found to emit light with Fluc. While the emission levels were weak compared to existing bioluminescent systems, mutant luciferases were identified that afforded enhanced outputs. The brightest luciferase-CouLuc-1 pair exhibited luminescent signal on par with native bioluminescent probes. Such robust emission suggests that the CouLuc-1 probes can be immediately adopted for biological imaging. The unique structural and optical features of the CouLuc-1 analogs are further well suited for multiplexed imaging. Combinations of red-emitting bioluminescent probes are necessary for applications in tissue and other scattering environments.

More broadly, our approach to accessing novel luminophores from simple fluorophores could spur the development of an expanded set of bioluminescent tools. Future work will investigate whether additional architectures can be accessed using the olefination strategy. Additional luminophore tuning via restricted rotation and extended pi-conjugation will also be explored.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details on luciferin synthesis, Rosetta analysis, library development, analog screens, and bioluminescence imaging are included.

**AUTHOR INFORMATION**
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SUPPORTING MATERIAL FOR

Coumarin luciferins and mutant luciferases for robust multicomponent bioluminescence imaging

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Figure S5. Biochemical analyses of Fluc with CouLuc-1 analogs. (a) Kinetics studies revealed CouLuc-1 analogs were poor binders of Fluc. (b) Kinetic constants shown are apparent values, determined via measurements of the initial rates of light emission over a range of substrate concentrations. [a]Values were normalized to emission of Fluc/D-luciferin. Error bars represent the standard error of the mean for n = 3 experiments. [b]Kinetic parameters could not be determined due to low levels of light production.
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Table S1. Bioluminescence and fluorescence emission of CouLuc-1 analogs.

| Luciferin   | BL $\lambda_{\text{max}}$ (nm) (with Fluc) | FL $\lambda_{\text{max}}$ (nm) pH 7.4 PBS | FL $\lambda_{\text{max}}$ (nm) MeOH | FL $\lambda_{\text{max}}$ (nm) DMF |
|-------------|------------------------------------------|-----------------------------------------|-----------------------------------|-----------------------------------|
| CouLuc-1-NMe$_2$ | 620                                       | 627                                      | 650                               | 609                               |
| CouLuc-1-NH$_2$ | 597                                       | 593                                      | 588                               | 476                               |
| CouLuc-1-OH   | 625                                       | 600                                      | 541                               | 644                               |

Table S2. Fluorescence excitation and emission maxima for CouLuc-1 analogs.

| Luciferin   | Structure                  | Solvent  | $\lambda_{\text{abs}}$ (nm) | $\lambda_{\text{em}}$ (nm) |
|-------------|----------------------------|----------|-----------------------------|-----------------------------|
| CouLuc-1-NMe$_2$ | ![Structure](image1.png)  | MeOH     | 430                         | 650                         |
|             |                            | Dioxane  | 425                         | 479                         |
|             |                            | DMF      | 425                         | 609                         |
|             | ![Structure](image2.png)  | pH 7.4   | 436, 515                    | 627                         |
|             |                            | pH 3.0   | 528                         | 631                         |
|             |                            | pH 10.0  | 425                         | 564                         |
| CouLuc-1-NH$_2$ | ![Structure](image3.png)  | MeOH     | 418                         | 588                         |
|             |                            | Dioxane  | 406                         | 430                         |
|             |                            | DMF      | 417                         | 476                         |
|             | ![Structure](image4.png)  | pH 7.4   | 414                         | 593                         |
|             |                            | pH 3.0   | 480                         | 607                         |
|             |                            | pH 10.0  | 405                         | 500, 582                    |
| CouLuc-1-OH  | ![Structure](image5.png)  | MeOH     | 442                         | 541                         |
|             |                            | Dioxane  | 388                         | 541                         |
|             |                            | DMF      | 394                         | 644                         |
|             | ![Structure](image6.png)  | pH 7.4   | 395, 479, 517               | 600                         |
|             |                            | pH 3.0   | 442                         | 517, 600                    |
|             |                            | pH 10.0  | 422                         | 600                         |
Figure S7. Spectroscopic properties of CouLuc-1 scaffolds. Normalized excitation (blue) and emission (red) spectra of CouLuc-1 analogs (10 µM) in MeOH (A-C), dioxane (D-F), DMF (G-I), and aqueous buffers (J-R). All three coumarin luciferins exhibit solvent-dependent fluorescence.
Figure S8. The binding pocket of Fluc does not accommodate the CouLuc-1 architecture. (a) CouLuc-1-NMe$_2$ was docked into the Fluc active site (PDB: 4G36) using the RosettaMatch algorithm. Residues within 5 Å of the bound luciferin are highlighted in orange. (b-c) Zoom-in view of (a). The coumarin portion of the luciferin is located near residues adjacent to C4' in bound D-luc.
Figure S9. Searching for a complementary luciferase via Rosetta-guided library design. (a) Residues within 6 Å of the docked CouLuc-1-NMe₂ scaffold were subjected to RosettaDesign. From the analysis, 40 residues were mutated (orange). (b-c) Zoom-in view of (a). Active site residues sculpted to accommodate the CouLuc-1 structures mitigating the steric clash observed in Figure S8b was mitigated. (d). From the analysis, 20 of the 40 residues mutated by Rosetta were targeted for library construction.
Figure S10. Evolving a brighter luciferase for CouLuc-1-NMe₂ via RosettaDesign. (a) Functional mutants identified from on-plate screens were picked and subjected to two secondary screens. In the first round, luciferase expression was autoinduced and mutants with >10-fold light emission (compared to Fluc) were re-examined via IPTG induction. (b) Variants with reproducible improvements were considered hits and sequenced. (c) Unique sequences identified from (b). Plasmids encoding mutant hits were isolated and re-introduced to E. coli. The magnitude of improvement was re-analyzed in a final assay using IPTG induction. Relative light emissions are plotted as fold over the native enzyme. Error bars represent the standard error of the mean for n = 3 experiments.

Figure S11. Evolving a brighter luciferase for CouLuc-1-OH via RosettaDesign. (a) Functional mutants identified from on-plate screens were picked and subjected to two secondary screens. In the first round, luciferase expression was autoinduced and mutants with >10-fold light emission (compared to Fluc) were re-examined via IPTG induction. (b) Variants with reproducible improvements were considered hits and sequenced. (c) Unique sequences identified from (b). Plasmids encoding these mutants were isolated and re-introduced to E. coli. The magnitude of improvement was re-analyzed in a final assay using IPTG induction. Relative light emissions are plotted as fold over the native enzyme. Error bars represent the standard error of the mean for n = 3 experiments.
Figure S12. Identifying complementary luciferases for CouLuc-1 analogs via semi-rational library design. (a-c) CouLuc-1-NH₂ and (d-e) CouLuc-1-OH were screened against a panel of mutant luciferases using a protocol from Rathbun, et al., with some modifications. Bacteria harboring the luciferase gene were induced for protein expression in a 96 deep-well plate. The cells were pelleted and resuspended in phosphate buffer (250 mM sodium phosphate, pH 8). Each luciferin was added (100 μM) and the plate was imaged, and the luminescent values for each mutant were referenced to native Fluc. Mutants with >5-fold improvement in flux (black) with (b) CouLuc-1-NH₂ or (e) CouLuc-1-OH were classified as “hits” and their sequences were listed in (c) and (f), respectively.
Figure S13. Improved light emission was recapitulated with recombinant Pecan (a) Light emission of CouLuc-1 analogs (250–2.5 µM) when incubated with ATP (1 mM), coenzyme A (1 mM) and recombinant Pecan (160 nM). Emission intensities are plotted as total photon flux values. Error bars represent the standard error of the mean for n = 3 experiments. (b) Tabulated photon outputs from (a) with [luciferin] = 100 µM. Fold improvements for each analog with Pecan (compared to Fluc) are also listed.

Figure S14. Biochemical analyses of Fluc with CouLuc-1 analogs. (a) Kinetics studies revealed CouLuc-1 analogs were poor binders of native luciferase. (b) Kinetic constants are apparent values, determined via measurements of initial light emission over a range of substrate concentrations. [a]Values were normalized to emission of Fluc/D-luciferin. Error bars represent the standard error of the mean for n = 3 experiments. [b]Kinetic parameters could not be determined due to low photon outputs.
Figure S15. Red-shifted bioluminescence was maintained with Pecan. (a) Recombinant Pecan was incubated with CouLuc-1 analogs and emission spectra were recorded. (b) Emission maxima ($\lambda_{em}$) for each analog. The corresponding emission maxima with Fluc are also shown.

| Lucifer | Fluc $\lambda_{max,em}$ | Pecan $\lambda_{max,em}$ |
|---------|-------------------------|--------------------------|
| o-luciferin | 556                    | N.D.                     |
| CouLuc-1-NMe$_2$ | 620            | 625                     |
| CouLuc-1-NH$_2$ | 597             | 590                     |
| CouLuc-1-OH  | 625             | 640                     |

Figure S16. Cellular imaging with Pecan and CouLuc-1-NMe$_2$. HEK293 cells ($5 \times 10^4$) expressing Fluc or Pecan were incubated with CouLuc-1-NMe$_2$ (250–2.5 μM) or D-luciferin (250–2.5 μM). Transfection efficiencies were determined via co-expression of GFP. (a) Maximum photon outputs ([luciferin] = 250 μM) were determined by monitoring signals over time. (b) Peak emission intensities for each probe combination are shown as photon flux values per cell. Error bars represent the standard error of the mean for n = 3 experiments. (c) Tabulated photon outputs from (b) with [luciferin] = 250 μM. Relative emission values for each luciferase/luciferin pair (compared to Fluc/D-luciferin) are also listed.

| Lucifer | Luciferase | Raw flux       | Transfection efficiency (% GFP) | Radiance per cell (p/s) | Relative emission |
|---------|------------|----------------|---------------------------------|-------------------------|-------------------|
| D-luciferin | Fluc       | $6.6 \pm 0.29 \times 10^7$ | $51 \pm 0.40\%$                   | $2.6 \pm 0.12 \times 10^3$ | 100 ± 4.4%         |
| CouLuc-1-NMe$_2$ | Fluc       | $4.6 \pm 0.43 \times 10^7$ | $51 \pm 0.40\%$                   | $1.8 \pm 0.17 \times 10^3$ | 69 ± 7.2%          |
| CouLuc-1-NMe$_2$ | Pecan      | $5.0 \pm 0.40 \times 10^8$ | $39 \pm 0.72\%$                   | $2.5 \pm 0.21 \times 10^4$ | 960 ± 90%          |
**Figure S17. Improved light emission observed with Pecan and other CouLuc-1 analogs in cellulo.** HEK293 cells (5 x 10^4) expressing Fluc or Pecan were incubated with CouLuc-1-NH₂ (250–2.5 µM), CouLuc-1-OH (250–2.5 µM) or D-luciferin (250–2.5 µM). Transfection efficiencies were determined via co-expression of GFP. (a) Peak emission intensities for each probe combination are shown as photon flux values per cell. Error bars represent the standard error of the mean for n = 3 experiments. (b) Tabulated photon outputs from (a) with [luciferin] = 250 µM. Relative emission values for each luciferase/luciferin pair (compared to Fluc/D-luciferin) are also listed.

| Luciferin        | Luciferase | Raw flux  | Transfection efficiency (% GFP) | Radiance per cell | Relative emission |
|------------------|------------|-----------|--------------------------------|-------------------|-------------------|
| D-luciferin      | Fluc       | 6.6 ± 0.29 x 10^7 | 51 ± 0.40%                      | 2.6 ± 0.12 x 10^3 | 100 ± 4.4%        |
| CouLuc-1-NH₂     | Fluc       | 2.7 ± 0.48 x 10^7 | 51 ± 0.40%                      | 1.1 ± 0.19 x 10^2 | 4.1 ± 0.74%       |
| CouLuc-1-OH      | Fluc       | 3.5 ± 0.40 x 10^4 | 51 ± 0.40%                      | 1.4 ± 0.16        | 0.052 ± 0.0065%   |
| CouLuc-1-NH₂     | Pecan      | 7.3 ± 0.25 x 10^7 | 39 ± 0.72%                      | 3.7 ± 0.14 x 10^3 | 142 ± 8.3%        |
| CouLuc-1-OH      | Pecan      | 2.6 ± 0.13 x 10^6 | 39 ± 0.72%                      | 1.3 ± 0.71 x 10^2 | 5 ± 0.35%         |

**Figure S18. Robust light emission observed in DB7 cells.** DB7 cells stably expressing Pecan (5 x 10^4) were incubated with CouLuc-1 analogs (250–2.5 µM). Photon outputs were measured immediately post substrate addition. Emission intensities are plotted as total photon flux values and error bars represent the standard error of the mean for n = 3 experiments.
Figure S19. Cellular light emission from Pecan/CouLuc-1-NMe₂ is comparable to other red-emitting bioluminescence probes. HEK293 cells (5 x 10⁴) expressing mutant luciferases or Fluc were incubated with either D-luciferin (250–2.5 µM), CouLuc-1-NMe₂ (250–2.5 µM), CycLuc1 (250–2.5 µM) or AkaLumine (250–2.5 µM). (a) Peak emission intensities for each probe combination are shown as photon flux values per cell. Error bars represent the standard error of the mean for n = 3 experiments. (b) Tabulated photon outputs from (a) with [luciferin] = 250 µM. Relative emission values for each luciferase/luciferin pair (compared to Fluc/D-luciferin) are also listed.

| Luciferin | Luciferase | Raw flux | Transfection efficiency (% GFP) | Radiance per cell | Relative emission |
|-----------|------------|----------|---------------------------------|-------------------|-------------------|
| D-luciferin     | Fluc       | 6.6 ± 0.29 x 10⁷ | 51 ± 0.40%                      | 2.6 ± 0.12 x 10³ | 100 ± 4.4%        |
| CouLuc-1-NMe₂  | Pecan      | 5.0 ± 0.40 x 10⁸ | 39 ± 0.72%                      | 2.5 ± 0.21 x 10⁴ | 960 ± 90%         |
| CycLuc1        | Fluc       | 1.2 ± 0.10 x 10⁸ | 51 ± 0.40%                      | 4.7 ± 0.40 x 10³ | 180 ± 18%         |
| AkaLuc         | AkaLumine  | 5.7 ± 0.20 x 10⁷ | 20 ± 0.49%                      | 5.7 ± 0.20 x 10³ | 218 ± 76%         |

Figure S20. Pecan/CouLuc-1-NMe₂ produces a significant amount of near infrared photons. Luciferase-expressing DB7 cells (5 x 10⁴) were treated with either CouLuc-1-NMe₂ (100 µM), D-luciferin (100 µM), CycLuc1 (100 µM), AkaLumine (100 µM) or furimazine (1:100 dilution from commercial stock). Photons produced in the near-infrared window were recorded by measuring through a Cy5.5 emission filter (695–770 nm). Emission intensities are plotted as total photon flux values and error bars represent the standard error of the mean for n = 3 experiments.
Figure S21. Multiplexed imaging with Pecan/CouLuc-1-NMe₂. Gradients of DB7 cells (1-4 x 10⁴) expressing Pecan, Akaluc, or Antares were plated in a 96-well plate as shown. (a) Raw luminescent images from sequential substrate administration of CouLuc-1-NMe₂ (100 µM), AkaLumine (100 µM), and furimazine (1:100 dilution from commercial stock). Data are representative of n = 3 replicates. (b) Quantified photon outputs for the images in (a). Photon flux from wells containing a single population of 4.0 x 10⁴ luciferase-expressing cells were plotted. Minimal crosstalk was observed between Pecan/CouLuc-1-NMe₂ and Akaluc/AkaLumine. Error bars represent the standard error of the mean for n = 3 experiments.
General biological methods

Fluorescent spectra and assays
Absorption curves were obtained on a Shimadzu UV-2550 spectrophotometer operated by UVProbe 2.32 software. Fluorescence traces were recorded on a PTI QuantaMaster steady state spectrofluorometer operated by FelixGX 4.2.2 software, with 5 nm excitation and emission slit widths, 0.1 s integration rate, and enabled emission correction. Data analyses and curve fitting were performed using MS Excel 2019 and GraphPad Prism 8. Luciferins (10 µM) were analyzed in a variety of solvents.

Bioluminescence emission spectra with recombinant luciferases
Emission spectra for all luciferin analogs were recorded on an Agilent Cary Eclipse Fluorescence Spectrophotometer. Each luciferin (100 µM) was incubated in an Eppendorf tube with ATP (1 mM) and diluted to 1 mL with bioluminescence reaction buffer (20 mM Tris•HCl, 0.5 mg/mL BSA, 0.1 mM EDTA, 1 mM TCEP, 2 mM MgSO₄, pH = 7.8). Purified luciferase enzyme (6–600 µg) was added, and an aliquot (700 µL) was transferred to a 10 mm pathlength cuvette. The emission slit widths were set to 5–10 nm. The detector gain was set to 600 mV. Emission data were collected at 1 nm intervals from 400–850 nm at ambient temperature. The acquisition times were set to 1–60 s/wavelength depending on the amount of light produced from each sample. Light emission was recorded as relative luminescence units (RLU), and the intensities were normalized. Area under the curve was estimated via the trapezoid rule on MS Excel.

Bioluminescence emission spectra with luciferase expressing cells
For in cellulo emission spectra, 1 x 10⁶ DB7 cells stably expressing the luciferase of interest⁵-⁶ were added to an Eppendorf tube in DMEM with 10% FBS. Cells were then incubated with luciferin (200 µM final concentration) diluted in PBS or DMSO (7-NMe2-CouLuc1 only, 10% DMSO final concentration) and an aliquot (700 µL) was transferred to a 10 mm pathlength cuvette. The emission slit widths were set to 20 nm. The detector gain was set to 800 mV. Emission data were collected at 5 nm intervals from 400–850 nm at ambient temperature. The acquisition times were set to 1 s/wavelength for all samples. Light emission was recorded at relative luminescence units (RLU) and the intensities were normalized. Area under the curve was estimated via the trapezoid rule on MS Excel.

Reagents
All reagents purchased from commercial supplies were of analytical grade and used without further purification. 4’-MorphoLuc, 7’-MorpipLuc, 7’-DMAMeLuc and 7’-pyrLuc, CycLuc1 were prepared and used as previously described⁵-⁷-⁸.

General bioluminescence imaging protocol
All analyses were performed in black 96-well plates (Grenier Bio One). Plates containing luminescent reagents were allowed to sit at room temperature for 5 min post-luciferin addition, and were then imaged in a light-proof chamber with an IVIS Lumina II (Xenogen) CCD camera chilled to –90 °C. The stage was kept at 37 °C during the imaging session, and the camera was controlled using Living Image software. The exposure time was 1–60 s, and data binning levels were set to medium. Regions of interests were selected for quantification and total flux values were
analyzed using Living Image software. All data were exported to Microsoft Excel or Prism (GraphPad) for further analyses.

**General cell culture methods**
HEK and DB7 cells were cultured in complete media: DMEM (Corning) containing 10% (v/v) fetal bovine serum (FBS, Life Technologies), 4.5 g/L glucose, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL, Gibco). DB7 cells stably expressing Fluc were generated according to Jones *et al.* via transduction with ecotropic retrovirus (Phoenix packaging system). For DB7 cells stably expressing Pecan, Akaluc, and Antares were generated according to Rathbun *et al.* via CRISPR-mediated gene insertion. For transient transfection experiments, HEK293 cells were plated 24–48 h prior to transfection in tissue culture treated 6-well dishes (Corning). Transfections were performed with luc2-IRE-eGFP or Pecan-IRE-eGFP plasmids using Lipofectamine 2000 according to the manufacturer’s instructions when cells were 75-80% confluent (1-2 d post plating). Cells were manipulated 24-48 h post transfection. Expression of all transient and stable cell lines were checked via flow cytometry using an ACEA NovoCyte flow cytometer and the appropriate filter settings. Fluorescence was analyzed and quantified using the NovoExpress software (ACEA). Stably expressing luciferase cells were maintained under puromycin selection (20 µg/mL) to ensure gene incorporation was preserved. Cells were incubated at 37 °C in a 5% CO2 humidified chamber. Cells were serially passaged using trypsin (0.25% in HBSS, Gibco).

**General cloning methods**
Polymerase chain reaction (PCR) methods were performed to isolate the luciferase and IRE-eGFP genes. Mutant luciferase inserts were amplified from pET vectors using the following primers:

5' - CGACTCACTATAGGAGACCCAAGCTTATGGAAGATGCCAAAAACATTAAGAAG -3' and
5' - CACCGGCCTTTATTCCAAGCGGCTTCGGCCAGTAACGTTTACACGGCGATCTTGCC -3'

IRES-eGFP insert was amplified from pcDNA vectors using the following primers:

5' - AAGGGCGGCAAGATCGCGGTAAAACGTTACTGCGCAAGCCGCTTGGAATAAG -3' and
5' - GCCGCCAGTGTGTAGGATATGATCTCGCAAGATTTACCTTTATCGACAGTCACTCATGC -3'

All PCR reactions (unless otherwise stated) were performed in a BioRad C3000 Thermocycler using the following conditions: 1) 95 °C for 3 min, 2) 95 °C for 30 s, 3) Tm of primers for 30 s, 4) 72 °C for 3 min, repeat steps 2-4 twenty times, then 72 °C for 5 min, and hold at 12 °C until retrieved from the thermocycler. Linearized vectors were generated via digestion with restriction enzymes *Hind*III and *Xho*I (New England BioLabs). The linearized vectors were combined with the appropriate luciferase insert by Gibson assembly (50 °C for 60 min). A portion of the reactions (3.0 µL) was directly transformed into TOP10 competent *E. coli* cells. Colonies containing the genes of interest were expanded overnight in 5 mL LB broth supplemented with ampicillin (100 µg/mL) or kanamycin (100 µg/mL) and DNA was extracted from colonies using a Zymo Research Plasmid Mini-prep Kit. Sequencing analysis confirmed successful plasmid generation.

**In cellulo bioluminescence imaging**
Stably expressing luciferase cells, or transiently transfected HEKs were plated in DMEM containing 10% FBS (90 µL, 50,000 cells/well). Measurements were carried out in triplicate using black 96-well plates (Grenier Bio One). Luciferin analogs (0–250 µM) were prepared as a 10X
stock in PBS and then 10 µL was added to assay wells. Images for all assays were acquired as described above.

**Construction of combinatorial codon mutagenesis (CCM) libraries**

DNA inserts for the combinatorial libraries (on average 3–4 mutations per clone) were generated as described by Belsare, et al., with some modifications. The library template was first amplified using primers ZY040 and ZY041 (Table S4). The following thermal cycling conditions was used in a BioRad C3000 Thermocycler: 1) 95 °C for 3 min, 2) 95 °C for 30 s, 3) 65 °C for 30 s, 4) 72 °C for 45 s min, repeat steps 2-4 twenty times, then 72 °C for 5 min, and hold at 12 °C until retrieved from the thermocycler.

The forward fragment reactions were performed using an equimolar mixture of mutagenic forward primers and ZY041 (Table S4). The reverse fragment reactions were performed using an equimolar mixture of mutagenic reverse primers and ZY040 (Table S4). The following thermal cycling conditions were used for the fragmentation reaction: 1) 95 °C for 3 min, 2) 95 °C for 30 s, 3) 60 °C for 30 s, 4) 72 °C for 45 s min, repeat steps 2-4 seven times, then 72 °C for 5 min, and hold at 12 °C until retrieved from the thermocycler. These reactions were used in a joining PCR reaction using the following conditions: ZY040 (1 µL, 100 µM), ZY041 (1 µL, 100 µM), 10x Q5® Reaction Buffer (6 µL), 10x Q5® GC Enhancer Buffer, 1:4 dilution of the forward fragment reaction (4 µL), 1:4 dilution of the reverse fragment reaction (4 µL), dNTPs (1 µL, 0.8 mM), and Q5® High-Fidelity DNA polymerase (0.3 µL, 1U, New England BioLabs) totaling 30 µL. DNA was amplified using thermal cycling conditions for insert amplification as described above. This PCR product was used as template for the second round of fragmentation (12 cycles) and joining PCRs. Mutagenesis was confirmed using Sanger sequencing (Genewiz).

Library DNA inserts were incorporated into linearized pQE vector. The linearized pQE vector was generated via digestion with restriction enzymes BamHI and XbaI. Library inserts were assembled with the linearized pET vectors using Gibson assembly. For each assembly, 25 ng of the linearized vector was combined with insert (5:1 insert:vector ratio) and added to 5 µL of master mix mixed with 5 µL NanoPure H₂O. The mixtures were incubated at 50 °C for 60 min, then the entire reaction mixture was transformed into chemically competent TOP10 E. coli (70 µL). Transformants were recovered with SOC (100 µL) for 30 mins at 37 °C and 25 µL plated per square, agar plate containing ampicillin.
Table S4: Primers used to construct Rosetta CCM library. The bases highlighted in red denote sites targeted for mutagenesis.

| Forward CCM Primers   | ZY040                                                                 | RosCCM1-F-218       | CGTTGTGTCCndtTTCACTGATGCC  |
|-----------------------|-----------------------------------------------------------------------|---------------------|----------------------------|
|                       | ATCGCATCACCATCACCATCACCATCACGGATCCATGGAAGATGCCAAAAACATTAAAGG         | RosCCM1-F-221      | CATTGAACndtGCGCGCG         |
|                       |                                                                       | RosCCM1-F-222      | GATTCACTGATCGCCGATACCCATCG  |
|                       |                                                                       | RosCCM1-F-229      | GACCCCATCTCCGCAGndtCAGCATC  |
|                       |                                                                       | RosCCM1-F-245      | GCCATTTCAACndtGGCTTCGSCAT  |
|                       |                                                                       | RosCCM1-F-246      | CATTTCACCACndtTCCGCACTTG  |
|                       |                                                                       | RosCCM1-F-247      | CACCCAGGGndtGCGCATGTC      |
|                       |                                                                       | RosCCM1-F-250      | CTTCGGCATGndtACCAGGTCG     |
|                       |                                                                       | RosCCM1-F-251      | CTTCGGCATGndtACCAGGTCG     |
|                       |                                                                       | RosCCM1-F-254      | TACCATCGCTGndtTACCTGATCTG  |
|                       |                                                                       | RosCCM1-F-314      | GATCGCCndtGGCGG            |
|                       |                                                                       | RosCCM1-F-338      | GGCGCCGGndtGCGTACCG        |
|                       |                                                                       | RosCCM1-F-342      | AGGCTACCGGndtACAGAAACA    |
|                       |                                                                       | RosCCM1-F-343      | CTACGCGCTGndtGAAACAATCTG  |
|                       |                                                                       | RosCCM1-F-347      | TGACAGAAACAAACTGndtGCCATTCGTGATCACC  |
|                       |                                                                       | RosCCM1-F-351      | TGCCATTCTGndtACCCCGAG     |
|                       |                                                                       | RosCCM1-F-352      | CATTCGTGACndtCCGGAAGGG    |
|                       |                                                                       | RosCCM1-F-420      | GGCTCGACndtGGCGACATCG     |
|                       |                                                                       | RosCCM1-F-437      | TCATCGTGGAACndtTCCGAAAGCC |
|                       |                                                                       | RosCCM1-F-519      | TGACCGCCGndtTTGGAAGGC    |

| Reverse CCM Primers   | 2Y041                                                                 | RosCCM1-R-218      | GGCGATGATCGAAahnGACACAAAGC  |
|-----------------------|-----------------------------------------------------------------------|---------------------|----------------------------|
|                       | TTTTCGTTTTTTTGGATGCTCTCTAGATTACACGGCGATCTTTGGCCGCTCTTTTTTT          | RosCCM1-R-221      | CGCGGGCahnnACTGAATCG       |
|                       |                                                                       | RosCCM1-R-222      | GATGGGTCGCCahnnATGACTGATC  |
|                       |                                                                       | RosCCM1-R-229      | GATGATCTGahnnGCCGAAGATGCGGT  |
|                       |                                                                       | RosCCM1-R-245      | ATGCCGGAAGCCahnnGTGAAATGCG |
|                       |                                                                       | RosCCM1-R-246      | AACATGCGAAahnGTTGGTAATGG  |
|                       |                                                                       | RosCCM1-R-247      | GAAACTGCCahnnGCCGTGGTG     |
|                       |                                                                       | RosCCM1-R-250      | CAGCGTGGTahnCATCGCAAG     |
|                       |                                                                       | RosCCM1-R-251      | CCAGCGTahnGAAACTGCGGAAG   |
|                       |                                                                       | RosCCM1-R-254      | CAGATCAAGTAahnCAGCGTGGTG  |
|                       |                                                                       | RosCCM1-R-314      | CCCGCCahnnGCCGATC         |
|                       |                                                                       | RosCCM1-R-338      | CGTAGGCCahnnGCCGATGC     |
|                       |                                                                       | RosCCM1-R-342      | TTGTTTCTGTahnnGCCGTAGCCT  |
|                       |                                                                       | RosCCM1-R-343      | CACTAGTTGGTTCCAhnGACCGCGTAG |
|                       |                                                                       | RosCCM1-R-347      | GGGATGAGCAATGGGCahnnAGTTGTTCCTGATCAG  |
|                       |                                                                       | RosCCM1-R-351      | CTTCGGGGGTahnCAGATGCGCA  |
|                       |                                                                       | RosCCM1-R-352      | CCCCTCAGGahnnGATCGAATG    |
|                       |                                                                       | RosCCM1-R-420      | GCGATGTCGCCahnhGTGCAAGCC |
|                       |                                                                       | RosCCM1-R-437      | GGCTCTTACAGhnnGTCACAGATGA |
|                       |                                                                       | RosCCM1-R-519      | GGCGTCAhnnGCCGAGTG      |
**Primary screening protocol**
The aforementioned agar plates were sprayed with either a solution of CouLuc-1-NMe$_2$ or CouLuc-1-OH (100–500 µM, 500 µL per plate). The plates were incubated at 25 °C for 5 minutes and imaged as described above. Light emitting colonies were picked and grown for secondary screenings.

**Secondary screening protocol**
Hits from the primary screen were further analyzed as described in Jones, *et al.*, with some modifications. Light-emitting colonies from the agar plates were picked and expanded in LB broth containing ampicillin (100 µg/mL, LB-AMP) in a 96-well deep-well plate (500 µg/well). The plate was incubated at 37 °C overnight. An aliquot of the overnight culture (4 µL) was then used to inoculate 400 µL of auto-induction LB media, and the cells were incubated as 30 °C with shaking (250 rpm) for 24 h. The remaining starter cultures were mixed with 50% glycerol (1:1) and stored at −80 °C for subsequent plasmid recovery and sequencing analysis. The cells were pelleted by centrifugation (4000 rpm for 10 min) and resuspended in phosphate buffer (600 µL, 250 mM sodium phosphate, pH = 7.8). Bacterial culture (90 µL) was added to 96-well black plates, followed by a 10X solution of luciferin and ATP in phosphate buffer (10 µL, 250 mM phosphate, pH = 7.8, 100 µM luciferin and 1 mM ATP final concentration). The plate was then imaged as described above. Mutants with light emission 10-fold greater than wild type Fluc were considered as hits.

The panel of mutants from above was further validated in a second round of analysis. TOP10 E. coli cells expressing the desired mutants (glycerol stocks) were used to inoculate 5 mL LB-AMP media. The cultures were incubated at 37 °C overnight. An aliquot of the starter culture (150 µL) was used to inoculate a fresh solution of LB-AMP (5 mL) and incubated at 37 °C to mid-log phase (O.D.$_{600}$~0.8). The cultures were then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 500 µM final concentration), and incubated at 23 °C for 16–18 h. The cells were harvested by centrifugation at 3600 rpm for 15 min. The cells were pelleted by centrifugation (4000 rpm for 10 min) and resuspended in sodium phosphate buffer (600 µL, 100 mM, pH 8). Cell lysate was spread across six cells (90 µL/well) on six different 96-well black plates. Native Fluc expressing bacteria were included in each screen as a control for compound integrity. To each well, a 10X solution of luciferin and ATP in phosphate buffer (10 µL, 250 mM phosphate, pH = 7.8, 100 µM luciferin and 1 mM ATP final concentration). The plate was then imaged as described above. Mutants with reproducible improvement (>10-fold over Fluc) were sequenced.

**Complete analog/mutant luciferase screen**
The panel of luciferin analogs was screened against a library of functional luciferase mutants described in Rathbun, *et al.* BL21 *E. coli* cells expressing mutant luciferases (glycerol stocks) were used to inoculate LB-Kan media in a 96-well deep-well plate (500 µL/well). The plate was incubated at 37 °C overnight. An aliquot of the overnight culture (4 µL) was used to inoculate 400 µL of auto-induction LB media, and the cells were incubated at 30 °C with shaking (250 rpm) for 24 h. The cells were pelleted by centrifugation at 4000 rpm for 10 min and resuspended in sodium phosphate buffer (600 µL, 100 mM, pH 8). Cell lysate was spread across six cells (90 µL/well) on six different 96-well black plates. Native Fluc expressing bacteria were included in each screen as a control for compound integrity. To each well, a 10X solution of luciferin and ATP in phosphate buffer (10 µL, 250 mM phosphate, pH = 7.8, 250 µM luciferin and 1 mM ATP final concentration)
was added, and the plate was imaged as described above. This process was repeated until all compounds were imaged with all 222 luciferase mutants.

**Substrate unmixing analysis with orthogonal pairs**

Substrate unmixing was conducted using ImageJ (installed under the FIJI package) as described in Rathbun, *et al.*

Luminescence images containing raw photon counts were imported into FIJI and subjected to a 2-pixel median filter. Next, the signal at each pixel was min-max scaled to lie between 0 and 65535 (the maximum value that can be stored in a 16-bit image). Images were then stacked, and an additional image containing the maximum value of the stack was computed (as a Z projection). This new image was added to the stack, and signal was unmixed using the ImageJ plugin developed by Gammon *et al.*

Pseudocolors were assigned in FIJI through the “Merge Channels” tool.

**Recombinant protein expression and purification**

Luciferases were expressed and purified as described by Jones, *et al.*

The pET-luciferase plasmids (WT, Pecan) were transformed into chemically competent BL21 *E. coli* cells. The transformants were plated on agar plates containing kanamycin. Cells were expanded in LB-Kan at 37 °C overnight. The overnight culture (20 mL) was used to inoculate 1 L LB-Kan and incubated at 37 °C to mid-log phase (O.D.~0.8). The culture was then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 500 µM final concentration), and incubated at 22 °C for 16–18 h. The cells were harvested at 4 °C by centrifugation at 4000 rpm for 15 min. Cell pellets were resuspended in 40 mL of phosphate buffer (50 mM phosphate, 300 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride, pH = 7.4). Lysozyme (2 mg) was added, and the cells were sonicated and centrifuged at 10000 rpm for 1 h at 4 °C. WT Fluc and mutant luciferases were purified from clarified supernatants using nickel affinity chromatography (BioLogic Duo Flow Chromatography System, Bio-Rad). Proteins were dialyzed into a Tris-acetate buffer (25 mM Tris-acetate, 1 mM EDTA, and 0.2 mM ammonium sulfate, pH = 7.8) at 4 °C for 16 h. DTT (1 mM final concentration) and 15% glycerol were added to the dialyzed samples prior to storage at −20°C. Final protein concentrations were determined using absorbance at 280 nm using a JASCO V730 UV-vis spectrophotometer. SDS-PAGE was also performed to verify protein purify, and gels were stained with Coomassie R-250.

**Light emission assays with recombinant luciferase**

Bioluminescence assays were performed as described by Jones, *et al.* Measurements were carried out in triplicate, using solid black, flat-bottom, 96-well plates (Grenier Bio One). Assay wells contained purified Fluc (0 or 1 mg), luciferin analogs (0–250 µM), ATP (Sigma Aldrich, 1 mM), coenzyme-A (trilithium salt, NanoLight Technologies, 1 mM), and diluted with bioluminescence reaction buffer to a total volume of 100 mL. Luciferins and ATP were premixed in the wells prior to Fluc addition. Images for all assays were acquired as described above.

**Bioluminescence kinetic measurements**

Bioluminescence kinetics assays were performed as described by Jones, *et al.* with some modifications. Measurements were acquired on a Tecan F200 Pro injection port luminometer with a neutral density filter. Reactions were performed in black 96-well flat-bottom plates (Greiner). Solutions of luciferin analog in bioluminescence reaction buffer were prepared (0.2–100 µM analog), and 50 µL were added to each well. The luminescence from each well was measured for
1.5 s prior to the addition of Fluc or mutant in bioluminescence buffer with ATP. For wells containing D-luciferin, a 1.6 μM solution of enzymes (50 μL) was used. For other compounds, a 160 μM solution of enzyme (50 μL) was administered. Following the addition of enzyme, luminescence was recorded every 0.2 s over a 60 s period. Samples were analyzed in triplicate. The peak intensities were determined by averaging the five maximum photon outputs per run. K_M and relative k_cat values were determined using nonlinear regression analyses in Prism (GraphPad).

**General Rosetta methods**

All calculations were carried out using Rosetta master version 60589 SHA1 code: 8442bff4fb7bf2ccf4655e8d15276c9b4cfbbd0 using the ref15 score function.\(^\text{12}\)

**Preparing the scaffolds**

A high-resolution (2.62 Å) structure of *Photinus pyralis* luciferase (PDB ID: 4G36) was processed to remove water molecules, non-proteinogenic molecules and a second copy of the protein in the asymmetric unit. Mutations present in the Pecan and Akaluc scaffold were made using the prepared 4g36 scaffold. The structures were subjected to an energy minimization using the Rosetta relax protocol to prepare them for subsequent protocols\(^\text{13}\) with the following command line:

```
<Path to>/Rosetta/main/source/bin/relax.default.linuxgccrelease -s
<input_file> @<Path to>/relax.flags
```

The contents of relax.flags was:

- nstruct 1
- relax:default_repeats 5
- relax: constrain relax to start coords
- relax: coord constrain sidechains
- relax: ramp constraints false
- ex1
- ex2
- use input_sc
- flip_HNQ
- ignore unrecognized_res
- relax: coord_cst_stdev 0.5

**Preparing the CouLuc-1 ligands**

The CouLuc-1 ligands were built in Avogadro: an open-source molecular builder and visualization tool. Version 1.2.0. [http://avogadro.cc/\(^\text{14}\)] and subjected to an energy minimization using the UFF force field.\(^\text{15}\) The .mol2 files were converted to .params files for use in Rosetta using an internal script. The params files used in the RosettaMatch algorithm are as follows:

The CouLuc-1- NMe2 params file where LCC stands for CouLuc-1-NMe2 is as follows:

```
NAME LCC
IO STRING LCC Z
TYPE LIGAND
AA UNK
ATOM N7 Ntrp X -0.50
ATOM S2 S X -0.05
ATOM O1 OOC X -0.65
ATOM O2 OOC X -0.65
ATOM O3 OH X -0.55
ATOM C5 CH2 X -0.07
```
ATOM  C6  CH1   X   0.02
ATOM  O4  OH    X  -0.55
ATOM  C9  CH1   X   0.02
ATOM  N4  Npro  X  -0.26
ATOM  C11 aroC  X  -0.01
ATOM  N3  Nhis  X  -0.42
ATOM  C10 aroC  X  -0.01
ATOM  N2  Nhis  X  -0.42
ATOM  C13 aroC  X  -0.01
ATOM  N6  NH2O  X  -0.36
ATOM  H6  Hpol  X  0.54
ATOM  H7  Hpol  X  0.54
ATOM  C12 aroC  X  -0.01
ATOM  N5  Ntrp  X  -0.50
ATOM  C14 aroC  X  -0.01
ATOM  H15 Haro X  0.22
ATOM  H2  Hpol  X  0.54
ATOM  H14 Haro X  0.22
ATOM  C8  CH1   X   0.02
ATOM  O6  OH    X  -0.55
ATOM  C7  CH1   X   0.02
ATOM  O5  OH    X  -0.55
ATOM  H3  Hpol  X  0.54
ATOM  H11 Hapo X  0.20
ATOM  H12 Hapo X  0.20
ATOM  H13 Hapo X  0.20
ATOM  H9  Hapo X  0.20
ATOM  H4  Hapo X  0.20
ATOM  H5  Hapo X  0.20
ATOM  C4  COO   X  0.73
ATOM  C3  aroC  X  -0.01
ATOM  N1  Nhis  X  -0.42
ATOM  C1  aroC  X  -0.01
ATOM  C15 CH1   X  0.02
ATOM  C16 COO   X  0.73
ATOM  O8  OOC   X  -0.65
ATOM  C18 aroC  X  -0.01
ATOM  C19 aroC  X  -0.01
ATOM  C20 aroC  X  -0.01
ATOM  C17 aroC  X  -0.01
ATOM  H17 Haro X  0.22
ATOM  C25 CH1   X  0.02
ATOM  F1  F     X  -0.14
ATOM  F2  F     X  -0.14
ATOM  F3  F     X  -0.14
ATOM  C24 aroC  X  -0.01
ATOM  C23 aroC  X  -0.01
ATOM  C22 aroC  X  -0.01
ATOM  C21 aroC  X  -0.01
ATOM  H20 Haro X  0.22
ATOM  N8  Nhis  X  -0.42
ATOM  C26 CH3  X  -0.16
ATOM  H21 Hapo X  0.20
ATOM  H22 Hapo X  0.20
ATOM  H23 Hapo X  0.20
ATOM  C27 CH3  X  -0.16
ATOM  H24 Hapo X  0.20
ATOM  H25 Hapo X  0.20
ATOM  H26 Hapo X  0.20
ATOM  H19 Haro X  0.22
ATOM  H18 Haro X  0.22
ATOM  H16 Hapo X  0.20
ATOM  S1  S     X  -0.05
ATOM  C2  aroC  X  -0.01
ATOM  H1  Haro X  0.22
ATOM  O7  ONH2  X  -0.44
ATOM  H10 Hpol  X   0.54
BOND_TYPE  C1   C15  1
BOND_TYPE  C1   N1  4
BOND_TYPE  N1   C3  4
BOND_TYPE  O1   S2  2
BOND_TYPE  C1   S1  4
BOND_TYPE  C2   C3  4
BOND_TYPE  C2   H1  1
BOND_TYPE  N2   C10  4
BOND_TYPE  N2   C13  4
BOND_TYPE  O2   S2  2
BOND_TYPE  C2   O3  1
BOND_TYPE  S2   N7  1
BOND_TYPE  C3   C4  1
BOND_TYPE  N3   C10  4
BOND_TYPE  N3   C11  4
BOND_TYPE  O3   C5  1
BOND_TYPE  C4   N7  1
BOND_TYPE  N4   C9  1
BOND_TYPE  O4   C6  1
BOND_TYPE  C5   C6  1
BOND_TYPE  C5   H5  1
BOND_TYPE  N5   C4  1
BOND_TYPE  N5   C12  4
BOND_TYPE  N5   C14  4
BOND_TYPE  O5   C7  1
BOND_TYPE  C6   H7  1
BOND_TYPE  C6   C7  1
BOND_TYPE  C6   C8  1
BOND_TYPE  C6   H9  1
BOND_TYPE  C7   C8  1
BOND_TYPE  C7   H11  1
BOND_TYPE  C7   H12  1
BOND_TYPE  C8   C9  1
BOND_TYPE  C8   C10  1
BOND_TYPE  C8   C11  1
BOND_TYPE  C8   H1  4
BOND_TYPE  C9   H13  1
BOND_TYPE  C9   N4  4
BOND_TYPE  C10  C11  1
BOND_TYPE  C10  C12  1
BOND_TYPE  C10  C13  1
BOND_TYPE  C12  C13  1
BOND_TYPE  C13  C14  1
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BOND_TYPE  C15  C16  4
BOND_TYPE  C15  C17  4
BOND_TYPE  C16  C17  4
BOND_TYPE  C17  C18  4
BOND_TYPE  C17  C20  4
BOND_TYPE  C18  C19  4
BOND_TYPE  C18  C21  4
BOND_TYPE  C19  C20  4
BOND_TYPE  C19  C24  4
BOND_TYPE  C20  C25  4
BOND_TYPE  C21  C22  4
| BOND_TYPE | C21  | H20  | 1  |
|-----------|------|------|----|
| BOND_TYPE | N8   | C22  | 1  |
| BOND_TYPE | C22  | C23  | 4  |
| BOND_TYPE | C23  | C24  | 4  |
| BOND_TYPE | C23  | H19  | 1  |
| BOND_TYPE | C24  | H18  | 1  |
| BOND_TYPE | C25  | F1   | 1  |
| BOND_TYPE | C25  | F2   | 1  |
| BOND_TYPE | C25  | F3   | 1  |
| BOND_TYPE | C26  | H21  | 1  |
| BOND_TYPE | C26  | H22  | 1  |
| BOND_TYPE | C26  | H23  | 1  |
| BOND_TYPE | C27  | H25  | 1  |

**CHI 1**

- C8   | C7   | O5   | H3
- PROTON_CHI 1 SAMPLES: 21, 45, 55, 60, 65, 70, 75, -45, -50, -55, -60, -65, -70, -75, 165, 170, 175, 180, 185, 190, 195 EXTRA: 0

**CHI 2**

- C9   | C8   | O6   | H8
- PROTON_CHI 2 SAMPLES: 21, 45, 55, 60, 65, 70, 75, -45, -50, -55, -60, -65, -70, -75, 165, 170, 175, 180, 185, 190, 195 EXTRA: 0

**CHI 3**

- N1   | C1   | C15  | C16

**CHI 4**

- N7   | S2   | O3   | C5

**CHI 5**

- C4   | N7   | S2   | O1

**CHI 6**

- N7   | C4   | N1   | C3

**CHI 7**

- S2   | O3   | C5   | C6

**CHI 8**

- S2   | N7   | C4   | C3

**CHI 9**

- O4   | C9   | N4   | C11

**CHI 10**

- O3   | C5   | C6   | O4

**CHI 11**

- O4   | C6   | C5   | O3

**CHI 12**

- O3   | C6   | C5   | O4

**CHI 13**

- C1   | C15  | C16  | C17

**CHI 14**

- C16  | C17  | C18  | C19

**CHI 15**

- C20  | C21  | C22  | C23

**CHI 16**

- C23  | C22  | N8   | C26

**NBR_ATOM**

- N7

**NBR_RADIUS**

- 16.480287

**ICOOR_INTERNAL**

- N7    0.000000  0.000000  0.000000  N7  S2  O1
- S2    0.000000  180.000000  1.650062  S2  N7  O1
- O1   -110.996100  68.074449  1.445125  S2  N7  O1
- O3   -129.794407  59.837306  1.410327  S2  N7  O1
- C5   -150.220766  54.143952  1.365802  S2  N7  O1
- C6   48.026210  157.652992  1.410874  S2  N7  O1
- O4   125.140105  125.140105  1.328601  S2  N7  O1
- C9   179.435301  59.581414  1.322176  S2  N7  O1
- C11  -0.833691  57.581285  1.328601  S2  N7  O1
- C13  58.153548  179.85440  1.342096  S2  N7  O1
- C14  179.945780  40.016656  0.984393  S2  N7  O1
- C15  179.945780  40.016656  0.984393  S2  N7  O1
- C16  179.945780  40.016656  0.984393  S2  N7  O1
- C17  179.945780  40.016656  0.984393  S2  N7  O1
- C18  179.945780  40.016656  0.984393  S2  N7  O1
- C19  179.945780  40.016656  0.984393  S2  N7  O1
- C20  179.945780  40.016656  0.984393  S2  N7  O1
- C21  179.945780  40.016656  0.984393  S2  N7  O1
- C22  179.945780  40.016656  0.984393  S2  N7  O1
- C23  179.945780  40.016656  0.984393  S2  N7  O1
- C24  179.945780  40.016656  0.984393  S2  N7  O1
- C25  179.945780  40.016656  0.984393  S2  N7  O1
- C26  179.945780  40.016656  0.984393  S2  N7  O1
- C27  179.945780  40.016656  0.984393  S2  N7  O1
- C28  179.945780  40.016656  0.984393  S2  N7  O1
- C29  179.945780  40.016656  0.984393  S2  N7  O1
- C30  179.945780  40.016656  0.984393  S2  N7  O1

\[S27\]
The contents of the CouLuc-1-NH₂ ligand params file where LCD stands for CouLuc-1-NH₂ are as follows:

NAME LCD
IO STRING LCD Z
TYPE LIGAND
AA UNK
ATOM N7 Ntrp X -0.51
ATOM S2 S X -0.06
ATOM O1 OOC X -0.66
ATOM O2 OOC X -0.66
ATOM O3 OH X -0.56
ATOM C5 CH₂ X -0.08
ATOM C6 CH₁ X 0.01
ATOM O4 OH X -0.56
ATOM C9 CH₁ X 0.01
ATOM N4 Npro X -0.27
ATOM C11 arOC X -0.01
ATOM N3 NHᵢs X -0.43
ATOM C10 arOC X -0.01
ATOM N2 NHᵢs X -0.43
ATOM C13 arOC X -0.01
| Bond Type | Atom 1 | Atom 2 | Bond Order |
|-----------|--------|--------|------------|
| C3        | C4     | 1      |
| N3        | C10    | 4      |
| N3        | C11    | 4      |
| O3        | C5     | 1      |
| C4        | N7     | 1      |
| C4        | O7     | 2      |
| N4        | C9     | 1      |
| N4        | C11    | 4      |
| N4        | C14    | 4      |
| O4        | C6     | 1      |
| O4        | C9     | 1      |
| C5        | N7     | 1      |
| C5        | H5     | 1      |
| N5        | C12    | 4      |
| N5        | C14    | 4      |
| N5        | H2     | 1      |
| O5        | C7     | 1      |
| O5        | H3     | 1      |
| C6        | C7     | 1      |
| C6        | H9     | 1      |
| N6        | C13    | 1      |
| N6        | H6     | 1      |
| N6        | H7     | 1      |
| O6        | C8     | 1      |
| O6        | C9     | 1      |
| O6        | H8     | 1      |
| C7        | C8     | 1      |
| C7        | H11    | 1      |
| N7        | H10    | 1      |
| C8        | C9     | 1      |
| C8        | H12    | 1      |
| N8        | C13    | 1      |
| N8        | H21    | 1      |
| N8        | H22    | 1      |
| O8        | C18    | 4      |
| O8        | C9     | 1      |
| C9        | H13    | 1      |
| N9        | C14    | 1      |
| N9        | H15    | 1      |
| N9        | C16    | 1      |
| N9        | H16    | 1      |
| O9        | C17    | 1      |
| O9        | C18    | 1      |
| C10       | C14    | 1      |
| C10       | H15    | 1      |
| C10       | C16    | 1      |
| C10       | H16    | 1      |
| O10       | C17    | 1      |
| O10       | C18    | 1      |
| C11       | C12    | 1      |
| C11       | C13    | 4      |
| C11       | H15    | 1      |
| C11       | C16    | 1      |
| O11       | C17    | 1      |
| O11       | H17    | 1      |
| C12       | C18    | 1      |
| C12       | C19    | 1      |
| N12       | C13    | 4      |
| C12       | H17    | 1      |
| O12       | C18    | 1      |
| O12       | C19    | 1      |
| C13       | C20    | 1      |
| C13       | H18    | 1      |
| N13       | C14    | 1      |
| C13       | C21    | 1      |
| O13       | C15    | 1      |
| C13       | C22    | 4      |
| N13       | C16    | 1      |
| N13       | C17    | 1      |
| C14       | C15    | 1      |
| C14       | H18    | 1      |
| N14       | C16    | 1      |
| C14       | C17    | 1      |
| O14       | C18    | 1      |
| O14       | C19    | 1      |
| C15       | C20    | 1      |
| C15       | H18    | 1      |
| N15       | C16    | 1      |
| C15       | C21    | 1      |
| O15       | C17    | 1      |
| O15       | C18    | 1      |
| C16       | C20    | 1      |
| C16       | H18    | 1      |
| O16       | C17    | 1      |
| O16       | C18    | 1      |
| C17       | C20    | 1      |
| C17       | H18    | 1      |
| O17       | C18    | 1      |
| O17       | C19    | 1      |
| C18       | C20    | 1      |
| C18       | H18    | 1      |
| N18       | C19    | 1      |
| N18       | C20    | 1      |
| O18       | C19    | 1      |
| O18       | C20    | 1      |
| C19       | C20    | 1      |
| C19       | H18    | 1      |
| N19       | C20    | 1      |
| N19       | C20    | 1      |
| O19       | C20    | 1      |
| O19       | C20    | 1      |
| C20       | C20    | 1      |
| C20       | H18    | 1      |

CHI 1  C8  C7  O5  H3
PROTON CHI 1  SAMPLES 21 45 55 60 65 70 75 -45 -50 -55 -60 -65 -70 -75 165 170 175 180 185 190 195 EXTRA 0

PROTON CHI 2  SAMPLES 21 45 55 60 65 70 75 -45 -50 -55 -60 -65 -70 -75 165 170 175 180 185 190 195 EXTRA 0

CHI 3  N1  C1  C15  C16
CHI 4  N7  S2  O3  C5
CHI 5  C4  N7  S2  O1
CHI 6  N7  C4  N1
CHI 7  S2  O3  C5  C6
CHI 8  S2  N7  C4  C3
CHI 9  O4  C9  N4  C11
CHI 10  O3  C5  C6  O4
CHI 11  C1  C15  C16  O8
CHI 12  C19  C20  C25  F1
NBR_ATOM  N7
NBR_RADIUS 15.401802
ICOOR_INTERNAL    N7     0.000000    0.000000    0.000000   N7    S2    O1
ICOOR_INTERNAL    S2     0.000000  180.000000    1.643772   N7    S2    O1
ICOOR_INTERNAL    O1     0.000000   72.115214    1.437728   S2    N7    O1
ICOOR_INTERNAL    O2  -110.979907   68.095120    1.445125   S2    N7    O2
ICOOR_INTERNAL    O3  -129.796485   67.884564    1.508988   S2    N7    O3
ICOOR_INTERNAL    C5    47.972804   59.847207    1.411561   O3    S2    N7
ICOOR_INTERNAL    C6   157.660576   70.738957    1.511080   C5    O3    S2
ICOOR_INTERNAL    O4    77.333973   70.005558    1.402847   C6    C5    O3
ICOOR_INTERNAL    C9   125.086234   74.080093    1.413900   O4    C6    C5
ICOOR_INTERNAL    N4  -109.502643   70.720185    1.445332   C9    O4    C6
ICOOR_INTERNAL    C11  -150.229052   54.143952    1.365802   N4    C9    O4
ICOOR_INTERNAL    N3  -0.892936   46.223588    1.342216   C11   N4    C9
ICOOR_INTERNAL    C10  -179.432403   59.624923    1.326020   N3    C11   N4
ICOOR_INTERNAL    N2  -0.848435   57.610071    1.331554   C10   N3    C11
ICOOR_INTERNAL    C13    0.552053   58.120141    1.342096   N2    C10   N3
ICOOR_INTERNAL    N6  -179.890730   60.506917    1.449335   C13   N2    C10
ICOOR_INTERNAL    H6  -0.115371   59.977458    0.984393   N6    C13   N2
ICOOR_INTERNAL    H7  -179.952772   59.961946    0.984831   N6    C13   H6
ICOOR_INTERNAL    C8   125.086234   71.733653    1.475916   C9    O4    N4
ICOOR_INTERNAL    O6    90.149730   71.799851    1.374530   C8    C9    O4
ICOOR_INTERNAL    H8  -179.999645   70.557415    0.969220   O6    C8    C9
ICOOR_INTERNAL    C7   179.949517   44.621693    1.328601   C8    C9    O6
ICOOR_INTERNAL    C12  179.862428   62.184716    1.420651   C12   C11  N3
ICOOR_INTERNAL    N5  -179.949517   44.621693    1.328601   C12   C11  N4
ICOOR_INTERNAL    C14  179.677558   70.203038    1.324715   C12   C11  N5
ICOOR_INTERNAL    H15  -179.517871   55.041066    1.032119   C14   C12  N5
ICOOR_INTERNAL    H16  -179.953018   61.152494    1.031712   C12   C11  N6
ICOOR_INTERNAL    H17  -179.949517   55.041066    1.032119   C14   C12  H16
ICOOR_INTERNAL    C17    0.552053   58.120141    1.342096   C12   C11  C10
ICOOR_INTERNAL    C18    0.167837   61.905154    1.351574   C17   C19  C18
ICOOR_INTERNAL    H18  -0.558169   62.167587    1.515074   C18   C19  C17
ICOOR_INTERNAL    C19    0.167837   61.905154    1.351574   C18   C19  C17
ICOOR_INTERNAL    C20    0.167837   61.905154    1.351574   C18   C19  C17
ICOOR_INTERNAL    C21    0.167837   61.905154    1.351574   C18   C19  C17
ICOOR_INTERNAL    C22    0.167837   61.905154    1.351574   C18   C19  C17
ICOOR_INTERNAL    O1  -0.704200   65.114755    1.374596   C22   C21  C20
ICOOR_INTERNAL    O2  -120.759332   70.951926    1.385320   C22   C21  C20
ICOOR_INTERNAL    O3  -118.384368   70.903342    1.385320   C22   C21  C20
ICOOR_INTERNAL    O4  -119.946722   62.419046    1.414589   C22   C21  C20
ICOOR_INTERNAL    O5  -0.010362   59.023358    1.398205   C22   C21  C20
ICOOR_INTERNAL    O6    0.193614   59.392855    1.396772   C22   C21  C20

S31
The contents of the CouLuc-1-OH ligand params file where LCE stands for CouLuc-1-OH are as follows:

```
NAME LCE
IO_STRING LCE Z
TYPE LIGAND
AA UNK
ATOM N7  Ntrp  X  -0.50
ATOM S2   S    X  -0.05
ATOM O1   OOC   X  -0.65
ATOM O2   OOC   X  -0.65
ATOM O3   OH    X  -0.55
ATOM C5   CH2   X  -0.07
ATOM C6   CH1   X  0.02
ATOM O4   OH    X  -0.55
ATOM C9   CH1   X  0.02
ATOM N4   Npro  X  -0.26
ATOM C11  aroC  X  -0.00
ATOM N3   Nhis  X  -0.42
ATOM C10  aroC  X  -0.00
ATOM N2   Nhis  X  -0.42
ATOM C13  aroC  X  -0.00
ATOM N6   NH2O  X  -0.36
ATOM H6   Hpol  X  0.54
ATOM H7   Hpol  X  0.54
ATOM C12  aroC  X  -0.00
ATOM N5   Ntrp  X  -0.50
ATOM C14  aroC  X  -0.00
ATOM H15  Haro X  0.23
ATOM H2   Hpol  X  0.54
ATOM H14  Haro X  0.23
ATOM C9   CH1   X  0.02
ATOM O6   OH    X  -0.55
ATOM H8   Hpol  X  0.54
ATOM C7   CH1   X  0.02
ATOM O5   OH    X  -0.55
ATOM H3   Hpol  X  0.54
ATOM H11  Hapo X  0.21
ATOM H12  Hapo X  0.21
ATOM H13  Hapo X  0.21
ATOM H9   Hapo X  0.21
ATOM H4   Hapo X  0.21
ATOM H5   Hapo X  0.21
ATOM C4   COO   X  0.73
ATOM C3   aroC  X  -0.00
ATOM N1   Nhis  X  -0.42
ATOM C1   aroC  X  -0.00
ATOM C15  CH1   X  0.02
ATOM C16  COO   X  0.73
ATOM O8   OOC   X  -0.65
```
BOND_TYPE C8 C9 1
BOND_TYPE C8 H12 1
BOND_TYPE C8 C18 4
BOND_TYPE C9 H13 1
BOND_TYPE O9 H21 1
BOND_TYPE C10 H14 1
BOND_TYPE C11 C12 4
BOND_TYPE C12 C13 4
BOND_TYPE C14 H15 1
BOND_TYPE C15 C16 1
BOND_TYPE C15 H16 1
BOND_TYPE O8 C16 4
BOND_TYPE C16 C17 4
BOND_TYPE C17 C20 4
BOND_TYPE C17 H17 1
BOND_TYPE C18 C19 4
BOND_TYPE C18 C21 4
BOND_TYPE C19 C20 4
BOND_TYPE C19 C24 4
BOND_TYPE C20 C25 1
BOND_TYPE C21 C22 4
BOND_TYPE C21 H20 1
BOND_TYPE O9 C22 1
BOND_TYPE C22 C23 4
BOND_TYPE C23 C24 4
BOND_TYPE C23 H19 1
BOND_TYPE C24 C18 1
BOND_TYPE C25 F1 1
BOND_TYPE C25 F2 1
BOND_TYPE C25 F3 1

CHI 1 C8 C7 O5 H3

PROTON_CHI 1 SAMPLES 21 45 55 60 65 70 75 -45 -50 -55 -60 -65 -70 -75 165 170 175 180 185 190 195 EXTRA 0

CHI 2 C9 C8 O6 H8

PROTON_CHI 2 SAMPLES 21 45 55 60 65 70 75 -45 -50 -55 -60 -65 -70 -75 165 170 175 180 185 190 195 EXTRA 0

CHI 3 C23 C22 O9 H21

CHI 4 N1 C1 C15 C16
CHI 5 N7 S2 O3 C5
CHI 6 C4 N7 S2 O1
CHI 7 N7 C4 C3 N1
CHI 8 S2 O3 C5 C6
CHI 9 S2 N7 C4 C3
CHI 10 O4 C9 N4 C11
CHI 11 O3 C5 C6 O4
CHI 12 C1 C15 C16 O8
CHI 13 C19 C20 C25 F1

NBR_ATOM N7
NBR_RADIUS 15.401802

ICOOR_INTERNAL N7 0.000000 0.000000 0.000000 N7 S2 O1
ICOOR_INTERNAL S2 0.000000 180.000000 1.649480 N7 S2 O1
ICOOR_INTERNAL O1 0.000000 72.115214 1.437728 N7 S2 O1
ICOOR_INTERNAL O2 -110.979907 68.095120 1.445125 S2 N7 O1
ICOOR_INTERNAL O3 -129.796485 67.884564 1.508898 S2 N7 O2
ICOOR_INTERNAL C5 47.972804 59.847207 1.411561 O3 S2 N7
ICOOR_INTERNAL C6 157.660576 70.738957 1.511080 C5 O3 S2
ICOOR_INTERNAL O4 77.333973 70.005558 1.402847 C6 C5 O3
ICOOR_INTERNAL C9 125.086234 74.080093 1.413900 C6 C5 O4
ICOOR_INTERNAL N4 -109.502643 70.720185 1.445322 C9 O4 C6
ICOOR_INTERNAL N3 -0.892936 46.223588 1.342216 C11 N4 C9
ICOOR_INTERNAL C10 -179.432403 59.624923 1.326020 N3 C11 N4
ICOOR_INTERNAL N2 -0.848435 57.610071 1.331554 C10 N3 C11
ICOOR_INTERNAL C13 0.552053 58.120141 1.342096 N2 C10 N3
ICOOR_INTERNAL N6 -179.890730 60.506917 1.449335 C13 N2 C10
ICOOR_INTERNAL H6 -0.115371 59.977458 0.984393 N6 C13 N2

S34
The Fluc structure was used as input to the RosettaMatch protocol. This algorithm identifies potential binding modes of input ligands based on user-defined constraints. A binding interaction is considered a “hit” if the ligand atoms do not collide with the protein backbone atoms. The following command line was used to call the RosettaMatch application.

```
<Path to>/Rosetta/main/source/bin/match.linuxgccrelease -s <input_file> @<Path to>/general_match.flags
-match:scaffold active_site_residues_for_geomcsts <Path to>/pos_file <Path to>/CouLuc-1_ligand.flags
```

Where the contents of the pos_file was as follows.

```
N_CST 1
1: 308
```
The contents of the constraint file were as follows.

```plaintext
CST::BEGIN
NATIVE
   TEMPLATE:: ATOM_MAP: 1 atom_name: O7 C4 C3
   TEMPLATE:: ATOM_MAP: 1 residue3: LCC/LCD/LCE
   TEMPLATE:: ATOM_MAP: 2 atom_name: N CA C ,
   TEMPLATE:: ATOM_MAP: 2 residue1: G
   TEMPLATE:: ATOM_MAP: 2 is_backbone
   CONSTRAINT:: distanceAB: 4.30 1.50 80.0 1 1
   CONSTRAINT:: angle_A: 135.3 10.0 10.0 360. 1
   CONSTRAINT:: angle_B: 43.6 10.0 10.0 360. 1
   CONSTRAINT:: torsion_A: 10.7 10.0 10.0 360. 1
   CONSTRAINT:: torsion_AB: -160.7 10.0 10.0 360. 1
   CONSTRAINT:: torsion_B: -134.1 10.0 10.0 360. 1

ALGORITHM_INFO:: match
   CHI_STRATEGY:: CHI 1 EX_THREE_THIRD_STEP_STDDEVS
   CHI_STRATEGY:: CHI 2 EX_THREE_THIRD_STEP_STDDEVS
ALGORITHM_INFO::END
CST::END
```

The contents of the CouLuc-1_ligand.flags files were as follows.

- extra_res_fa <Path to>/CouLuc-1_ligand.params
- match:geometric_constraint_file <Path to>/CouLuc-1_ligand.cst
- match:lig_name LCC/LCD/LCE

The contents of the general_match.flags files was as follows.

- packing
- -ex1
- -ex2
- -ex2aro
- -exlar
- -extrachi_cutoff 0
- -use_input_sc true
- -database <Path to>/Rosetta/main/database/
- -match:filter_colliding_upstream_residues
- -match:filter_upstream_downstream_collisions
- -match:upstream_residue_collision_tolerance 0.95
- -match:updown_collision_tolerance 0.3
- -match:bump_tolerance 0.3
- -match_grouper SameSequenceAndDSPositionGrouper
- -match:grouper_downstream_rmsd 0.5
- -match:eucilb bin_size 0.5
- -match:euler_bin_size 5.0
- -output_format PDB
- -exclude_patches N_acetylated
- -consolidate_matches 1
- -output_matches_per_group 1
- -output_matches_only false
- -enumerate_ligand_rotamers
- -only Enumerate_non_match_redundant_ligand_rotamers
- -out::file::output_virtual

The pdb files generated in the matching run were then used as inputs for RosettaDesign calculations. The RosettaDesign algorithm is used to re-sculpt the pocket surrounding the docked luciferin analogue in order to remove clashing side chains and introduce new, productive
interactions with the ligand. The RosettaDesign application was called with the following command line:

```bash
<path to>/Rosetta/main/source/bin/rosetta_scripts.linuxgccrelease -s <input_file> -parser:protocol <path to>/enzdes.xml -nstruct 1 -jd2:ntrials 1 -database <path to>/Rosetta/main/database/ @<path to>/CouLuc-1_ligand.flags @<path to>/general.flags
```

The contents of the RosettaDesign general.flags file was as follows:

```
-run::preserve_header
-enzdes::minimize_ligand_torsions 7.0
-enzdes::detect_design_interface
-unmute_protocols.enzdes.EnzRepackMinimize
-packing::use_input_sc
-packing::extrachi_cutoff 1
-packing::ex1
-packing::ex2
-linemem_ig 10
-in:ignore_unrecognized_res
-ligand::old_estat
-jd2:enzdes_out
-nblist_autoupdate
-score:weights <path to>/Rosetta/main/database/scoring/weights/ref2015.wts
-enzdes::bb_min_allowed_dev 0.05
-no_his_his_pairE
```

The contents of the RosettaDesign enzdes.xml file was as follows:

```xml
<ROSETTASCRIPS>
  <TASKOPERATIONS>
    <DetectProteinLigandInterface name="dsgn_cuts_on" cut1="6" cut2="8" cut3="10" cut4="12" design="1"/>
    <DetectProteinLigandInterface name="dsgn_cuts_off" cut1="6" cut2="8" cut3="10" cut4="12" design="0"/>
    <RestrictResiduesToRepacking name="pack_only" residues="210,221"/>
  </TASKOPERATIONS>
  <SCOREFXNS>
    <ScoreFunction name="ref2015" weights="ref2015.wts"/>
  </SCOREFXNS>
  <MOVERS>
    # Add constraints to file
    AddOrRemoveMatchCsts name="addcst" cst_instruction=add_new
cstfile="../inputs/FAB.cst"/>
    <AddOrRemoveMatchCsts name="addcst" cst_instruction="add_new"/>
    <AddOrRemoveMatchCsts name="rmvcst" cst_instruction="remove" keep_covalent="1"/>
    # Optimize the pose per the cst file
    <EnzRepackMinimize name="cstopt" scorefxn_minimize="ref2015" cst_opt="1" design="0" repack_only="0" fix_catalytic="0" minimize rb="1" minimize_bb="1"
minimize_sc="1" minimize lig="1" min_in_stages="1" cycles="1"
task_operations="dsgn_cuts_off"/>
    # Design and repacking around the catalytic residues; keep the catalytic residues fixed in this instance.
    <EnzRepackMinimize name="dsgn" scorefxn_minimize="ref2015" cst_opt="0" design="1" repack_only="0" fix_catalytic="1" minimize rb="1" minimize_bb="1"
minimize_sc="1" minimize lig="1" min_in_stages="1" backrub="0" cycles="1"
task_operations="dsgn_cuts_on,pack_only"/>
```
Synthetic materials and methods

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using anhydrous solvents. All commercially obtained reagents were used as received. Flash column chromatography was performed using reversed phase (100 Å, 20-40 micron particle size, RediSep® Rf Gold® Reversed-phase C18 or C18Aq) on a CombiFlash® Rf 200i (Teledyne Isco, Inc.). High-resolution LC/MS analyses were conducted on a Thermo-Fisher LTQ-Orbitrap-XL hybrid mass spectrometer system with an Ion MAX API electrospray ion source in negative ion mode. Analytical LC/MS was performed using a Shimadzu LCMS-2020 Single Quadrupole utilizing a Kinetex 2.6 μm C18 100 Å (2.1 x 50 mm) column obtained from Phenomenex, Inc. Runs employed a gradient of 0→90% MeCN/0.1% aqueous formic acid over 4.5 min at a flow rate of 0.2 mL/min. ¹H NMR and ¹³C NMR spectra were recorded on Bruker spectrometers (at 400 or 500 MHz or at 100 or 125 MHz) and are reported relative to deuterated solvent signals. Data for ¹H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. Data for ¹³C NMR spectra are reported in terms of chemical shift. Absorption curves were obtained on a Shimadzu UV-2550 spectrophotometer operated by UVProbe 2.32 software. Fluorescence traces were recorded on a PTI QuantaMaster steady-state spectrofluorimeter operated by FelixGX 4.2.2 software, with 5 nm excitation and emission slit
widths, 0.1 s integration rate, and enabled emission correction. Data analysis and curve fitting were performed using MS Excel 2019 and GraphPad Prism 8.

**Synthetic procedures**

![Chemical structure diagram]

**General procedure for the synthesis of nitrile (2)**

To a solution of CH$_3$CN (8.0 mmol, 4.0 eq) in THF (20 mL) was added n-BuLi (8.0 mmol, 2.5 M, 4.0 eq) at -78 ºC. The solution was stirred at -78 ºC 10 minutes, after which a solution of coumarin (1) (2.0 mmol, 1.0 eq) in 5 mL of THF was added slowly. The reaction was stirred at -78 ºC for 10-15 min and quenched with 15 mL aqueous NH$_4$Cl solution. The mixture was warmed to room temperature and extracted with EtOAc and concentrated. To the crude oil was added 125 mL of 0.5 M HCl and stirred vigorously for 1-4 h. The precipitate was extracted with EtOAc, dried Na$_2$SO$_4$ and concentrated to give nitrile 2 as a mixture of isomers. Based on $^1$H NMR spectroscopic analysis, the resulting product was typically >90% pure and was typically used in the next step without further purification. Silica gel column chromatography could be performed using EtOAc/hexanes to obtain high purity material (>95%).

(Z/E)-2-(7-Dimethylamino)-4-(trifluoromethyl)-2H-chromen-2-ylidene)acetonitrile (2a).

Following the general procedure using commercial 7-(dimethylamino)-4-(trifluoromethyl)coumarin (1a) (514 mg, 2.0 mmol). Purification by flash chromatography on silica gel (hexanes/EtOAc, 0% to 20%) afforded 2a as a orange solid (358 mg, 64% yield). $^1$H NMR (CDCl$_3$, 400 MHz, compound exists as a mixture of isomers, Z-isomer denoted by *, E-isomer denoted by §) δ 7.32 – 7.26 (m, 1H*, 1H§), 6.85 (s, 1H§), 6.50 – 6.47 (m, 2H*, 1H§), 6.38 (s, 1H*), 6.33 (d, $J$ = 2.6 Hz, 1H§), 4.78 (s, 1H§), 4.48 (s, 1H*), 3.05 (s, 6H*), 3.04 (s, 6H§); $^{13}$C NMR (CDCl$_3$, 100 MHz) δ 164.1§, 162.6*, 154.6§, 153.1*, 131.7 (q, $J$ = 33.0 Hz)§, 131.1 (q, $J$ = 33.0 Hz)*, 125.8 (q, $J$ = 2.2 Hz)§, 125.6 (q, $J$ = 2.2 Hz)*, 122.3 (q, $J$ = 272.8 Hz)§, 122.2 (q, $J$ = 272.6 Hz)*, 117.7§, 116.9*, 112.6 (q, $J$ = 6.3 Hz)§, 111.2 (q, $J$ = 6.3 Hz)§, 108.7§, 108.7*, 103.4*, 103.3§, 98.7*, 98.1§, 73.6§, 72.3*, 40.2*, 40.2§; $^{19}$F NMR (CDCl$_3$, 377 MHz) δ -64.5§, -64.6*; HRMS (ESI) calculated for Z-isomer C$_{14}$H$_{12}$F$_3$N$_2$O (M+H)$^+$ 290.0896, observed 290.0896; E-isomer C$_{14}$H$_{12}$F$_3$N$_2$O (M+H)$^+$ 290.0896, observed 290.0901.
(Z/E)-2-(7-Amino-4-(trifluoromethyl)-2H-chromen-2-ylidene)acetonitrile (2b).
Following the general procedure using commercial 7-amino-4-(trifluoromethyl)coumarin (1b) (458 mg, 2.0 mmol). Purification by flash chromatography on silica gel (hexanes/EtOAc, 0% to 30%) afforded 2b as an orange solid (308 mg, 61% yield). 1H NMR (CD3CN, 400 MHz, compound exists as a mixture of isomers, Z-isomer denoted by *, E-isomer denoted by §) δ 7.24 – 7.18 (m, 1H*, 1H§), 6.77 (dd, J = 2.4, 1.2 Hz, 1H§), 6.62 (dd, J = 2.4, 1.2 Hz, 1H*), 6.51 – 6.49 (m, 1H*, 1H§), 6.44 (d, J = 2.3 Hz, 1H*), 6.37 (d, 1H§), 5.00 – 4.92 (m, 2H*, 2H§), 4.91 (s, 1H§), 4.73 (s, 1H*); 13C NMR (CD3CN, 125 MHz) δ 164.6§, 163.3*; 155.6*, 155.4*, 153.7§, 153.5*, 131.8 (q, J = 32.1 Hz)§, 130.7 (q, J = 32.1 Hz)*, 126.8 (q, J = 2.2 Hz)§, 126.6 (q, J = 2.2 Hz)*, 123.3 (q, J = 273.7 Hz)§, 123.3 (q, J = 273.5 Hz)*, 118.1§, 117.3*, 114.8 (q, J = 6.5 Hz)*, 112.2 (q, J = 6.5 Hz)§, 112.1*, 112.0§, 104.8*, 104.5*, 101.1*, 100.9§, 74.7§, 73.5*; 19F NMR (CD3CN, 377 MHz) δ -64.6*, -64.7§; HRMS (ESI) calculated for C12H8F3N2O (M+H) + 253.0583, observed 253.0582.

(Z/E)-2-(7-Hydroxy-4-(trifluoromethyl)-2H-chromen-2-ylidene)acetonitrile (2c).
Following the general procedure using commercial 7-hydroxy-4-(trifluoromethyl)coumarin (1c) (460 mg, 2.0 mmol). Purification by flash chromatography on silica gel (hexanes/EtOAc, 0% to 50%) afforded 2c as a yellow solid (354 mg, 70% yield). 1H NMR (CD3OD, 400 MHz, compound exists as a mixture of isomers, Z-isomer denoted by *, E-isomer denoted by §) δ 7.37 – 7.31 (m, 1H*, 1H§), 6.93 – 6.92 (m, 1H§), 6.86 – 6.84 (m, 1H*), 6.71 – 6.66 (m, 2H*, 1H§), 6.60 (d, J = 2.4 Hz, 1H§), 5.11 (s, 1H§), 4.96 (s, 1H*); 13C NMR (CD3OD, 100 MHz) δ 164.7§, 163.5*, 163.4§, 163.1*, 155.9§, 155.6*, 132.3 (q, J = 323.0 Hz)§, 131.1 (q, J = 323.0 Hz)*, 127.6*, 127.6§, 127.2 (q, J = 1.8 Hz)§, 127.0 (q, J = 1.8 Hz)*, 124.9*, 124.8§, 122.1*, 122.1§, 117.9*, 117.1 (q, J = 6.5 Hz)*, 117.0§, 114.3 (q, J = 6.4 Hz)§, 114.0§, 114.0*, 107.6*, 107.3§, 104.1§, 104.0*, 75.9§, 74.4*; 19F NMR (CD3OD, 377 MHz) δ -66.1§, -66.2*; HRMS (ESI) calculated for C12H3F3NO2 (M–H) − 252.0278, observed 252.0270.
General procedure for the synthesis of CouLuc-1-R

To a microwave vial containing nitrile (2) (0.15 mmol, 1.0 eq), D-cysteine hydrochloride monohydrate (0.23 mmol, 1.5 eq) and NaHCO$_3$ (0.60 mmol, 4.0 eq) was added degassed EtOH (1.5 mL). The suspension was heated at 85 ºC under N$_2$ and monitored by LC/MS. After 3-5 days the consumption of 2 is greater than 75%. The reaction mixture was cooled to room temperature and EtOH was evaporated under vacuum. The crude solid was triturated with Et$_2$O (3 x 5 mL), acidified to pH 1.0, filtered and wash with cold water (3 x 5 mL). The crude mixture was purified directly by reversed phase chromatography (C$_{18}$, 0-100% MeOH/water). The solvent was removed in vacuo to afford CouLuc-1-R.

(Z)-2-((7-(Dimethylamino)-4-(trifluoromethyl)-2H-chromen-2-ylidene)methyl)-4,5-dihydrothiazole-4-carboxylic acid (CouLuc-1-NMe$_2$).

Following the general procedure using 2a (42 mg, 0.15 mmol), CouLuc-1-NMe$_2$ was obtained as a red solid (23 mg, 40% yield). $^1$H NMR (CD$_3$OD + TFA-$d_1$, 500 MHz) $\delta$ 7.56 – 7.53 (m, 1H), 6.93 (s, 1H), 6.89 (dd, $J = 9.3, 2.6$ Hz, 1H), 6.76 (d, $J = 2.6$ Hz, 1H), 6.18 (s, 1H), 5.16 (dd, $J = 9.4, 5.6$ Hz, 1H), 4.00 – 3.90 (m, 2H), 3.14 (s, 6H); $^{13}$C NMR (125 MHz, DMSO-$d_6$ + TFA-$d_1$) $\delta$ 186.3, 179.5, 173.0, 164.0, 163.1, 143.1 (q, $J = 32.5$ Hz), 135.0, 131.6 (q, $J = 275.6$ Hz), 128.3, 122.4 (q, $J = 6.1$ Hz), 121.1, 112.9, 106.7, 102.8, 72.0, 43.4; $^{19}$F NMR (DMSO-$d_6$, 377 MHz) $\delta$ -63.4; HRMS (ESI) calculated for C$_{17}$H$_{15}$F$_3$N$_2$O$_3$S (M+H)$^+$ 385.0828, observed 385.0833.
(Z)-2-((7-Amino-4-(trifluoromethyl)-2H-chromen-2-ylidene)methyl)-4,5-dihydro-thiazole-4-carboxylic acid (CouLuc-1-NH₂).

Following the general procedure using 2b (37 mg, 0.15 mmol), CouLuc-1-NH₂ was obtained as an orange solid (28 mg, 52% yield). ¹H NMR (400 MHz, DMSO-d₆) δ 7.09 – 7.02 (m, 1H), 6.87 (s, 1H), 6.45 – 6.38 (m, 2H), 6.14 (s, 2H), 5.94 (t, J = 9.0 Hz, 1H), 4.96 (t, J = 9.0 Hz, 1H), 3.49 (dd, J = 11.1, 9.5 Hz, 1H), 3.41 (dd, J = 11.1, 8.5 Hz, 1H); ¹³C NMR (125 MHz, DMSO-d₆) δ 172.3, 162.5, 153.8, 152.8, 152.7, 126.5 (q, J = 30.6 Hz), 124.9, 122.5 (q, J = 271.9 Hz), 115.9 (q, J = 6.3 Hz), 110.3, 102.2, 101.8, 99.3, 76.2, 32.2; ¹⁹F NMR (DMSO-d₆, 377 MHz) δ -63.4; HRMS (ESI) calculated for C₁₅H₁₂F₃N₂O₃S (M+H)⁺ 357.0515, observed 357.0523.

(Z)-2-((7-hydroxy-4-(trifluoromethyl)-2H-chromen-2-ylidene)methyl)-4,5-dihydro-thiazole-4-carboxylic acid (CouLuc-1-OH).

Following the general procedure using 2c (38 mg, 0.15 mmol), CouLuc-1-OH was obtained as an orange solid (24 mg, 45% yield). ¹H NMR (500 MHz, CD₃OD + TFA-d₁) δ 7.63 – 7.61 (m, 1H), 7.25 (s, 1H), 7.03 (d, J = 2.4 Hz, 1H), 6.97 (dd, J = 8.9, 2.4 Hz, 1H), 6.32 (s, 1H), 5.29 (dd, J = 9.8, 5.7 Hz, 1H), 4.06 (dd, J = 12.1, 9.8 Hz, 1H), 4.01 (dd, J = 12.0, 5.7 Hz, 1H); ¹³C NMR (125 MHz, CD₃OD + TFA-d₁) δ 180.3, 170.6, 165.3, 164.7, 156.1, 136.4 (q, J = 33.2 Hz), 127.9 (q, J = 2.4 Hz), 123.2 (q, J = 272.3 Hz), 116.8 (q, J = 5.9 Hz), 116.4, 108.4, 104.3, 64.4, 35.1; ¹⁹F NMR (CD₃OD + TFA-d₁, 377 MHz) δ -65.5; HRMS (ESI) calculated for C₁₅H₁₁F₃NO₄S (M+H)⁺ 358.0355, observed 358.0358.
Synthesis of CouLuc-1-NMe₂ with chromatography-free procedure

![Chemical structure of 2a and CouLuc-1-NMe₂](image)

To a reaction flask containing nitrile (2a) (1.25 g, 4.49 mmol), d-cysteine hydrochloride monohydrate (1.18 g, 6.73 mmol) and NaHCO₃ (1.51 g, 17.94 mmol) was N₂-sparged EtOH (45 mL) was heated at 85 °C under N₂. After heating for 3 days the EtOH was evaporated under vacuum. The yellow solid was triturated with Et₂O (3 x 20 mL), acidified to pH 1.0 with 1M HCl to give a red solid that was separated by centrifugation and the supernatant was discarded. The precipitates were suspended in 15 mL water and then centrifuged. The washing process was repeated twice. The precipitate was dried under reduced pressure to afford CouLuc-1-NMe₂ as a red solid (468 mg, 27% yield) to provide high purity material by NMR (>95%).

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NMR Spectra

1H NMR
$^{13}$C NMR
$^1$H NMR
\( F_3C-\overset{\text{CN}}{\text{O}} \)

\( \text{NH}_2 \)

\[ 2b \]

\( ^{19}\text{F NMR} \)
$^{19}$F NMR
$^{19}$F NMR
$^{13}$C NMR

CouLuc-1-NH$_2$
CouLuc-1-OH

$^{13}$C NMR

- 180.3286
- 170.6241
- 165.3153
- 164.7077
- 156.0796
- 136.8947
- 127.8947
- 124.3325
- 122.1490
- 117.2725
- 116.7927
- 116.4453
- 108.3833
- 104.2889
- 95.0459
- 64.3672
- 35.0947
$^{19}$F NMR

CD$_3$OD + TFA-$d_i$
