SUPPORTING MATERIAL FOR

Coumarin luciferins and mutant luciferases for robust multicomponent bioluminescence imaging

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TABLE OF CONTENTS

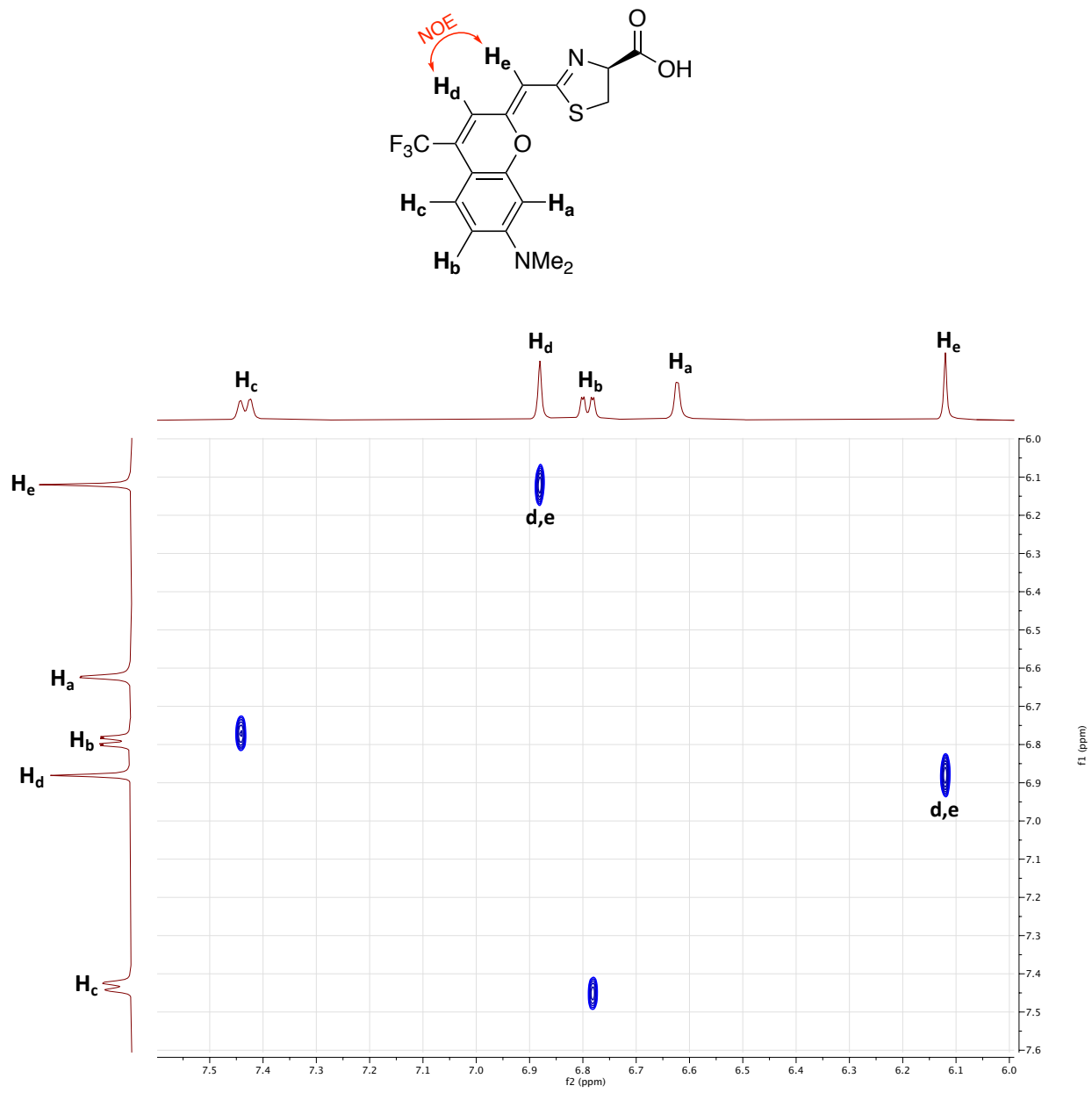
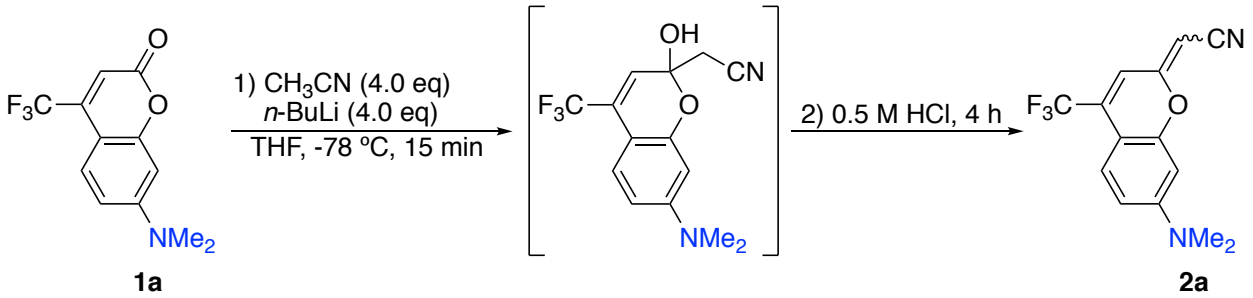


Figure S1. 2D NOESY spectrum (diagonal peak suppression) of CouLuc-1-NMe_2 in DMSO-d_6 showing the correlation of H_d and H_e.



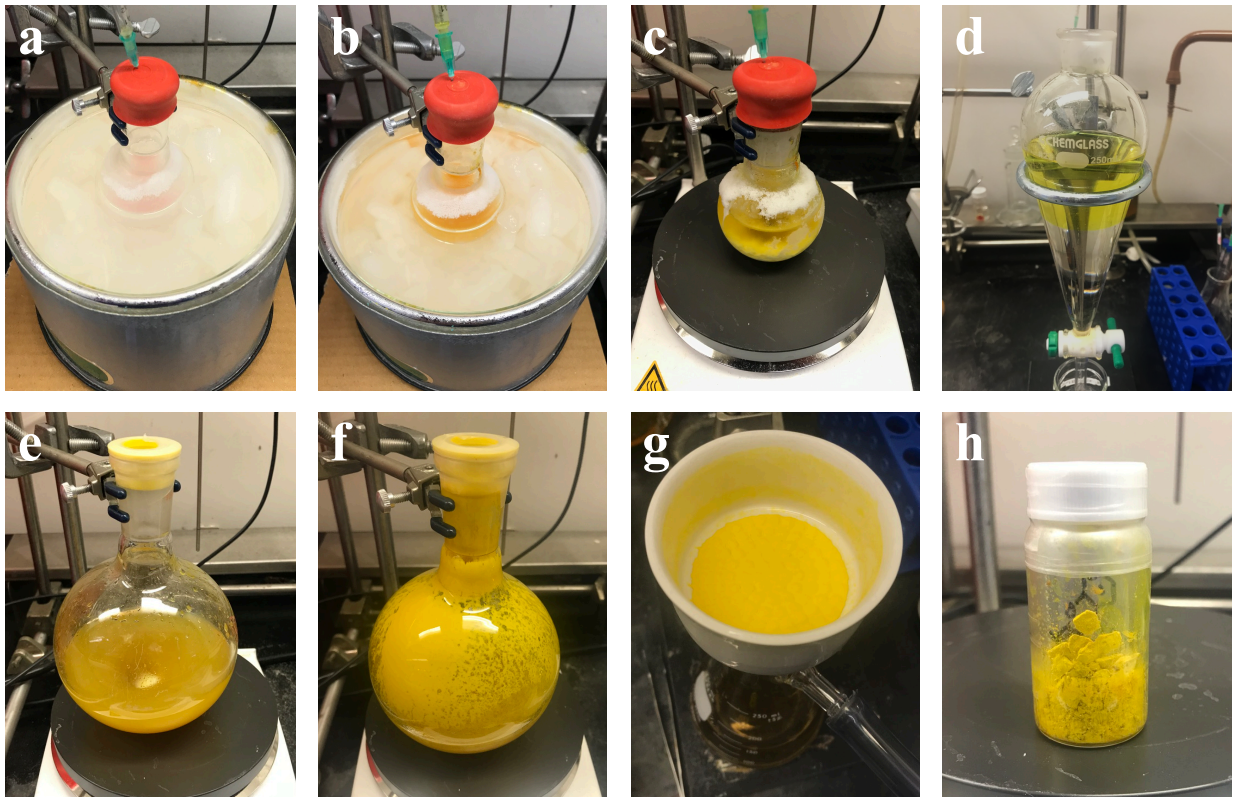
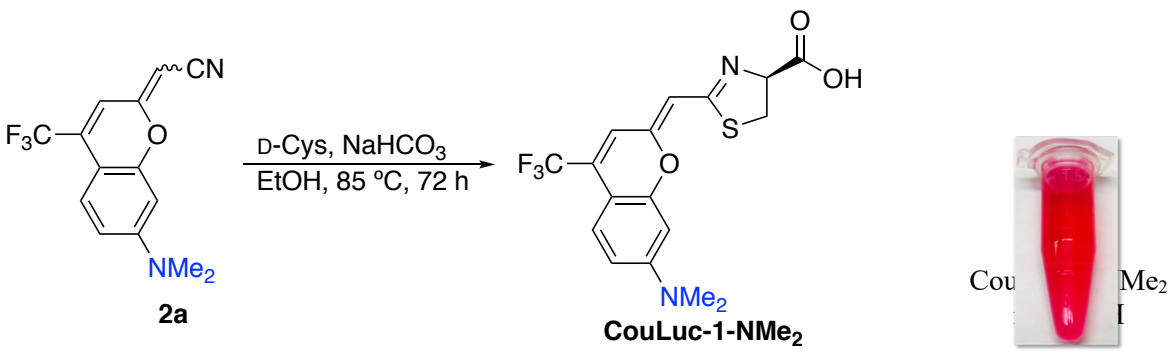


Figure S2. Chromatography-free synthesis of cyanomethylene coumarin 2a from commercial coumarin. Starting from a flame-dried round-bottom flask under nitrogen; (a) The flask was charged with THF, CH_3CN, and *n*-BuLi at –78 ºC; (b) After addition of coumarin **1a** in THF; (c) Reaction quenched with aqueous NH_4Cl solution and the –78 ºC bath was removed; (d) Extraction with EtOAc; (e) Addition of 0.5 M HCl and stirred at 1000 rpm; (f) Precipitation of cyanomethylene coumarin **2a** as a yellow solid; (g) Vacuum filtration; (h) Isolated cyanomethylene coumarin **2a**.



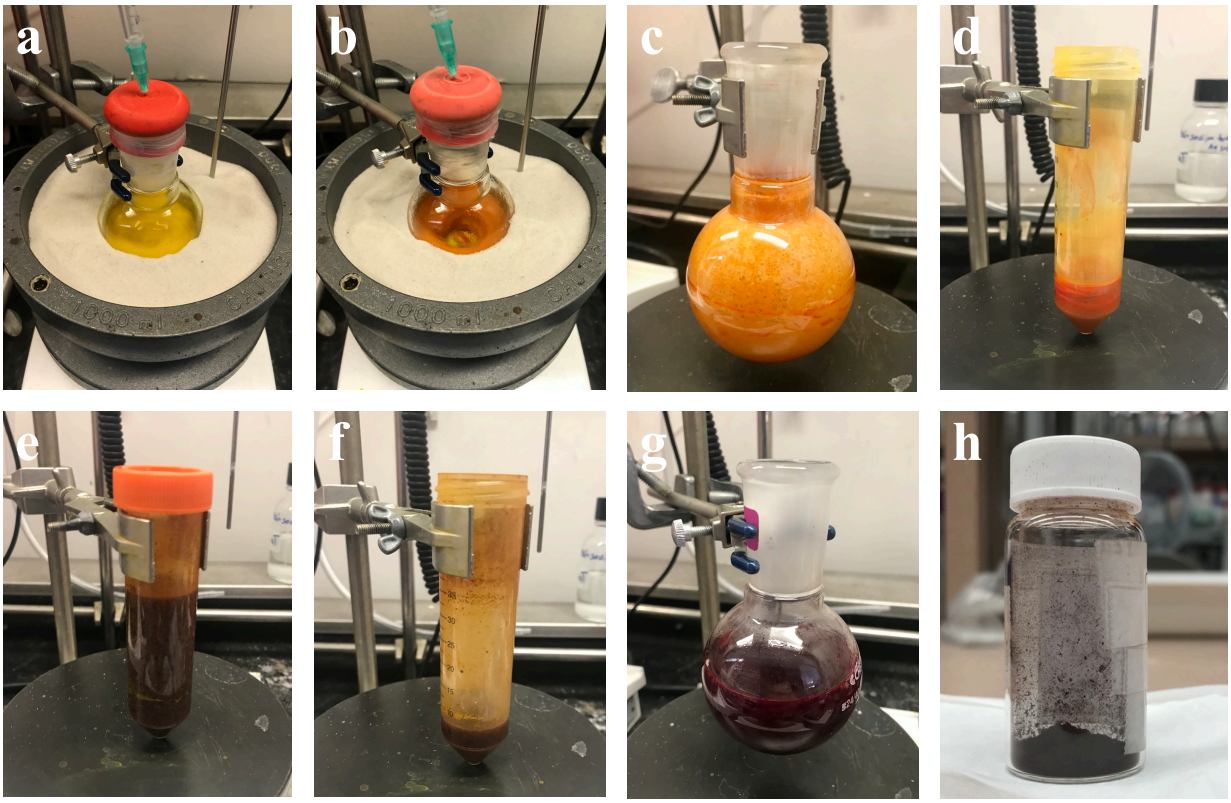
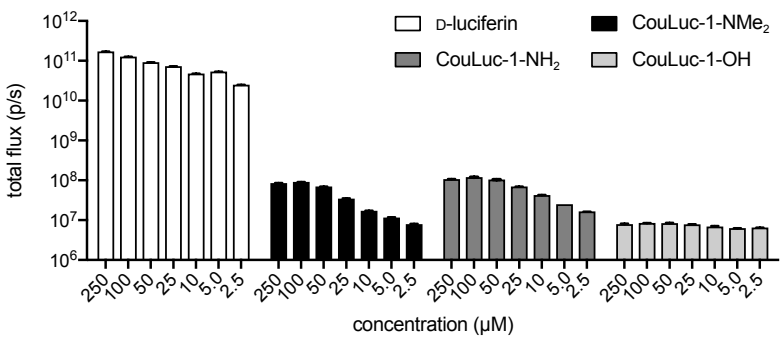


Figure S3. Chromatography-free synthesis of CouLuc-1-NMe$_2$ from cyanomethylene coumarin. Starting from a flame-dried round-bottom flask under nitrogen; (a) Flask charged with **2a**, D-cysteine, $NaHCO_3$ and degassed EtOH; (b) Reaction after >75% conversion; (c) Crude mixture after removing EtOH; (d) After triturating with EtOAc; (e) Color change after acidifying with 1.0 M HCl; (f) After centrifugation and supernatant removal; (g) Transferred product to round-bottomed flask with MeOH/H_2O, red color change; (h) Lyophilized CouLuc-1-NMe$_2$.

a

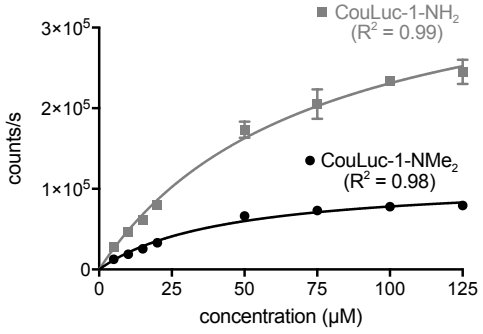


b

Luciferin	Photon output (p/s)	Relative emission (%)
D-luciferin	1.2 ± 0.001 x 10^{11}	100 ± 1.3
CouLuc-1-NMe_2	1.1 ± 0.02 x 10^{8}	9.4 ± 0.2 x 10^{-2}
CouLuc-1-NH_2	2.3 ± 0.05 x 10^{8}	1.8 ± 0.04 x 10^{-1}
CouLuc-1-OH	1.6 ± 0.02 x 10^{7}	1.3 ± 0.02 x 10^{-2}

Figure S4. **CouLuc-1 analogs are competent, albeit weak, emitters with Fluc.** (a) Light emission of CouLuc-1 analogs (2.5–250 μM) or D-luc (2.5–250 μM) when incubated with ATP (1 mM), coenzyme A (1 mM) and recombinant Fluc (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). Relative emission values for each analog (compared to D-luc) at 100 μM are also listed

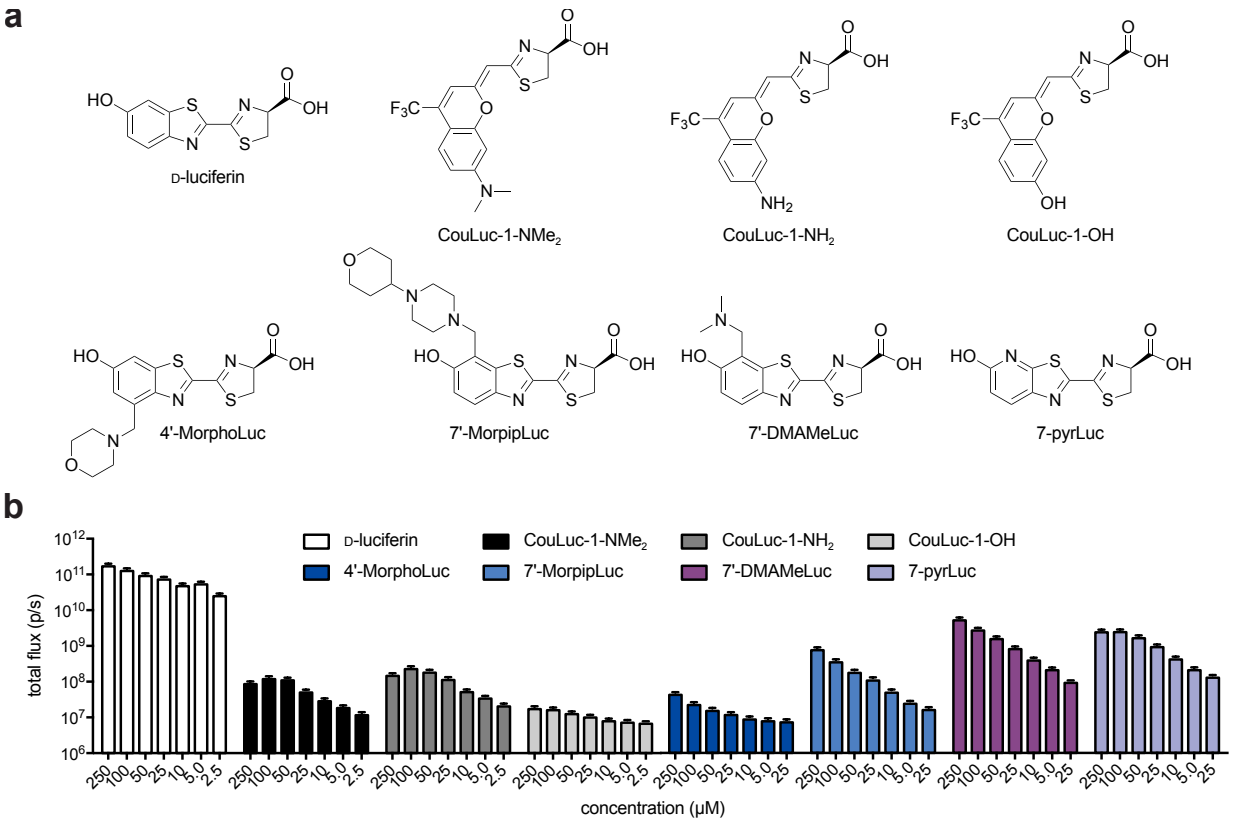
a



b

Luciferin	K_M (μM)	Normalized k_{cat}/K_M[a]
D-luciferin	20 ± 1.6	100 ± 12
CouLuc-1-NMe_2	43 ± 4.1	1.1 ± 0.16 x 10^{-2}
CouLuc-1-NH_2	72 ± 7.8	2.9 ± 0.50 x 10^{-2}
CouLuc-1-OH	_[b]	_[b]

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.



c

Luciferin	Photon output (p/s)	Relative emission (%)
D-luciferin	1.2 ± 0.001 x 10^{11}	100 ± 0.92
CouLuc-1-NMe$_2$	**1.1 ± 0.02 x 10^8**	**0.094 ± 0.002**
CouLuc-1-NH$_2$	**2.3 ± 0.05 x 10^8**	**0.18 ± 0.004**
CouLuc-1-OH	**1.6 ± 0.02 x 10^7**	**0.013 ± 0.0002**
4′-MorphoLuc	2.2 ± 0.04 x 10^7	0.018 ± 0.0004
7′-MorpipLuc	3.4 ± 0.1 x 10^8	0.27 ± 0.01
7′-DMAMeLuc	2.7 ± 0.06 x 10^9	2.12 ± 0.05
7′-pyrLuc	2.4 ± 0.04 x 10^9	1.92 ± 0.04

Figure S6. Comparison of CouLuc-1 analogs to other synthetic luciferins. (a) Chemical structures of the synthetic luciferins tested. (b) CouLuc-1 analogs or synthetic luciferins (2.5–250 μM) were incubated with ATP (1 mM), coenzyme A (1 mM) and recombinant Fluc (160 nM). Emission intensities are plotted as total photon flux values. Error bars represent the standard error of the mean for n = 3 experiments. (c) Tabulated photon outputs from (b) with [luciferin] = 100 μM. Relative emission values for each analog (compared to D-luciferin) are also listed.

Table S1. Bioluminescence and fluorescence emission of CouLuc-1 analogs.

Luciferin	BL λ_{max} (nm) (with Fluc)	FL λ_{max} (nm) pH 7.4 PBS	MeOH	DMF
CouLuc-1-NMe_2	620	627	650	609
CouLuc-1-NH_2	597	593	588	476
CouLuc-1-OH	625	600	541	644

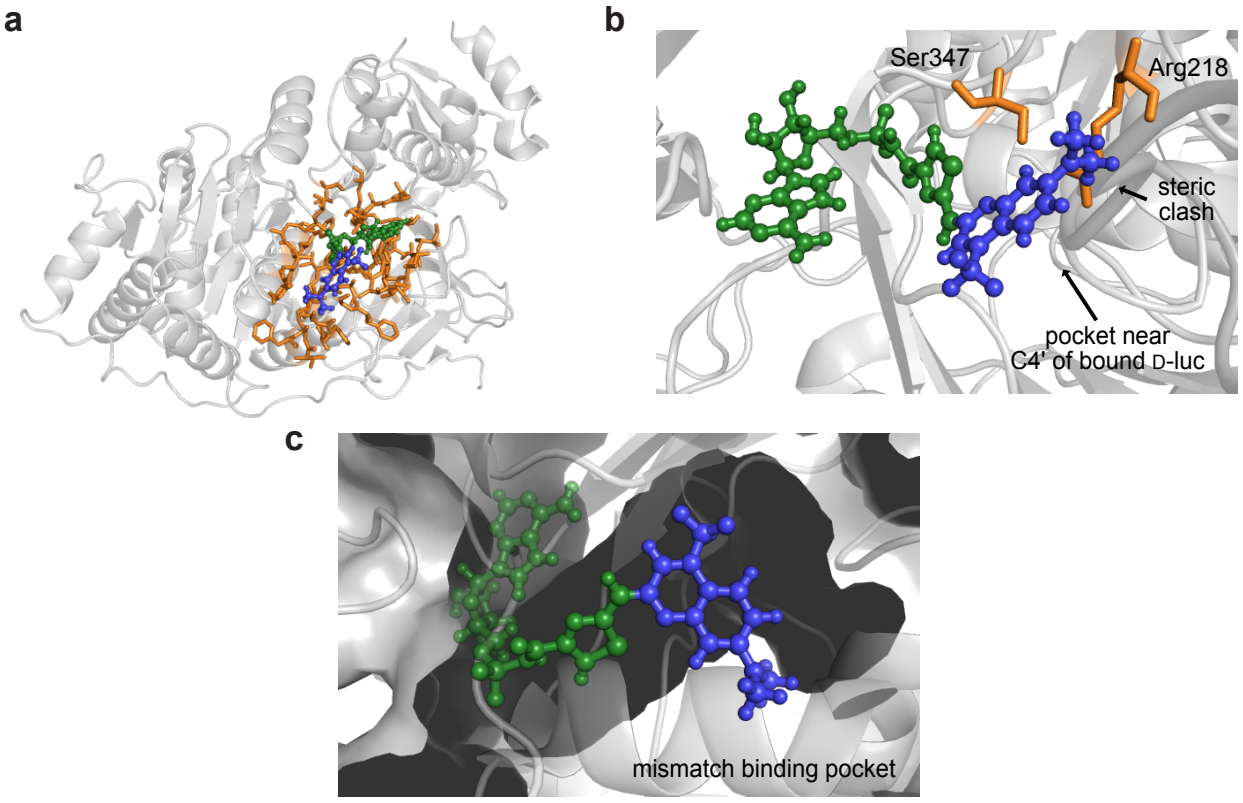
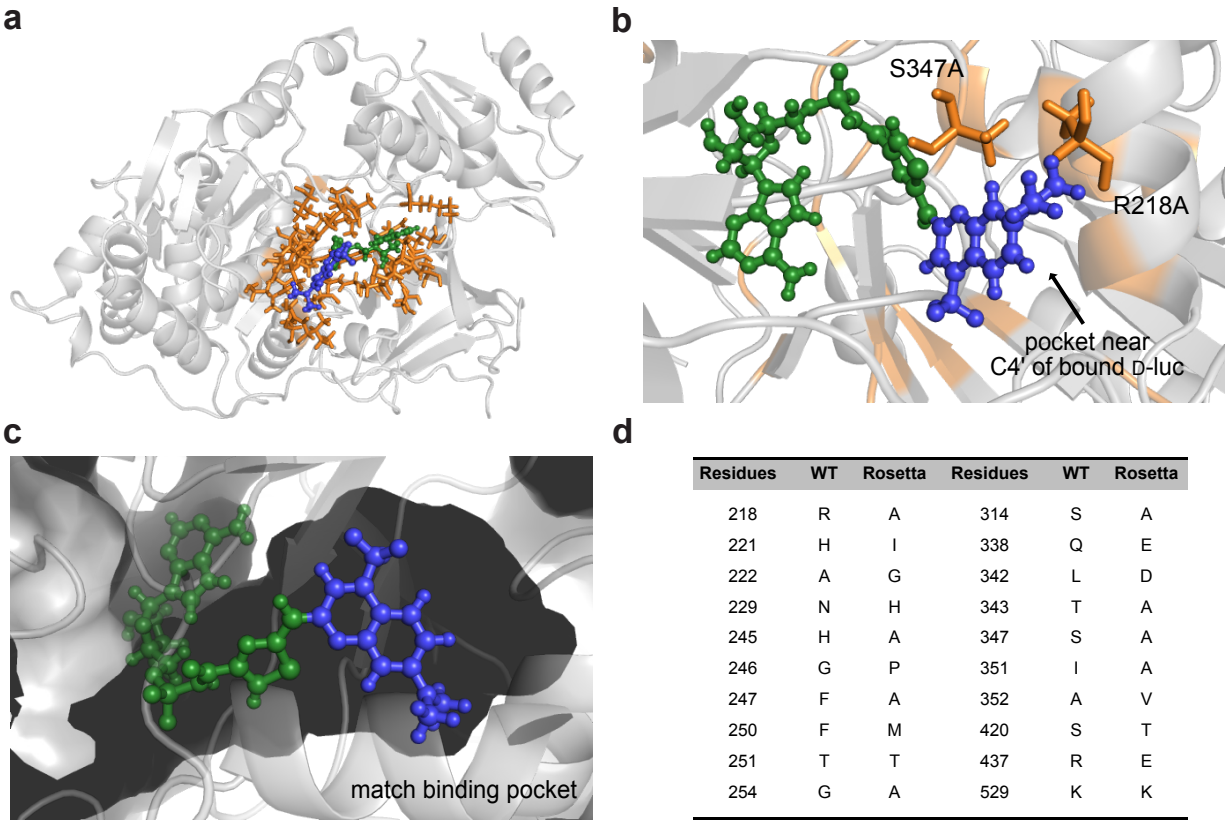


Figure S7. **The binding pocket of Fluc does not accommodate the CouLuc-1 architecture.** (a) CouLuc-1-NMe_2 was docked into the Fluc active site (PDB: 4G36) using the RosettaMatch algorithm.[1-2] Residues within 5 Å of the bound luciferin are highlighted in orange. (b-c) Zoom-in view of (a). The coumarin portion of the luciferin is located near residues adjacent to C4′ in bound D-luc.



Residues	WT	Rosetta	Residues	WT	Rosetta
218	R	A	314	S	A
221	H	I	338	Q	E
222	A	G	342	L	D
229	N	H	343	T	A
245	H	A	347	S	A
246	G	P	351	I	A
247	F	A	352	A	V
250	F	M	420	S	T
251	T	T	437	R	E
254	G	A	529	K	K

Figure S8. Searching for a complementary luciferase via Rosetta-guided library design. (a) Residues within 6 Å of the docked CouLuc-1-NMe_2 scaffold were subjected to RosettaDesign.[1-2] 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 S7b was mitigated. (d). From the analysis, 20 of the 40 residues mutated by Rosetta were targeted for library construction.

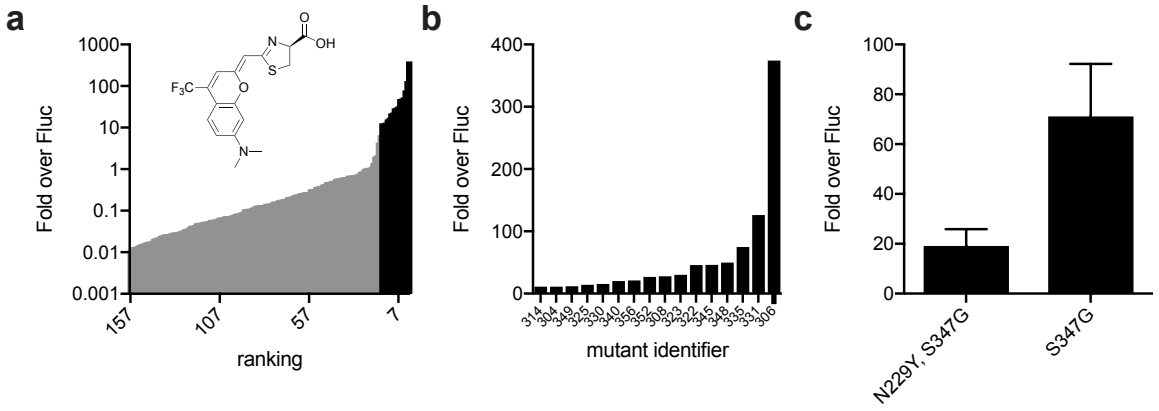


Figure S9. Evolving a brighter luciferase for CouLuc-1-NMe$_2$ via RosettaDesign. (a) Functional mutants identified from on-plate screens were picked and subjected to two secondary screens. In the first round, luciferase expression was auto-induced[3] and mutants with >10-fold light emission (compared 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.

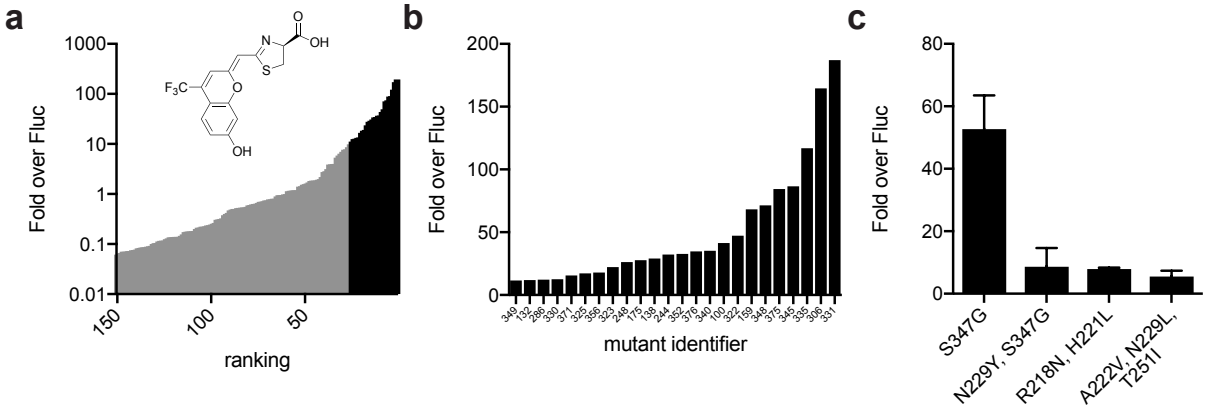
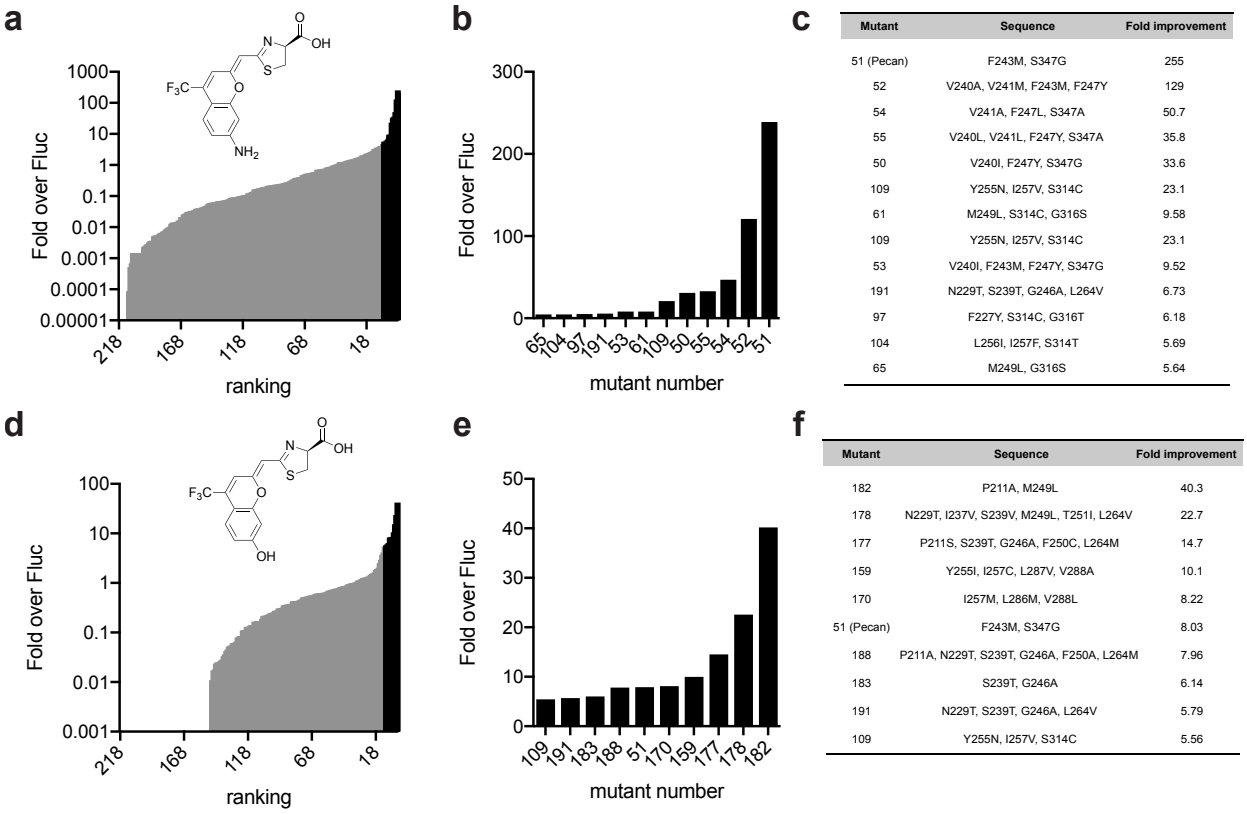


Figure S10. Evolving a brighter luciferase for CouLuc-1-OH via RosettaDesign. (a) Functional mutants identified from on-plate screens were picked and subjected to two secondary screens. In the first round, luciferase expression was autoinduced[3] and mutants with >10-fold light emission (compared to 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.

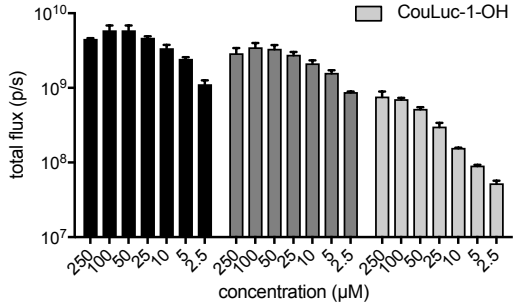


Mutant	Sequence	Fold improvement
51 (Pecan)	F243M, S347G	255
52	V240A, V241M, F243M, F247Y	129
54	V241A, F247L, S347A	50.7
55	V240L, V241L, F247Y, S347A	35.8
50	V240I, F247Y, S347G	33.6
109	Y255N, I257V, S314C	23.1
61	M249L, S314C, G316S	9.58
109	Y255N, I257V, S314C	23.1
53	V240I, F243M, F247Y, S347G	9.52
191	N229T, S239T, G246A, L264V	6.73
97	F227Y, S314C, G316T	6.18
104	L256I, I257F, S314T	5.69
65	M249L, G316S	5.64

Mutant	Sequence	Fold improvement
182	P211A, M249L	40.3
178	N229T, I237V, S239V, M249L, T251I, L264V	22.7
177	P211S, S239T, G246A, F250C, L264M	14.7
159	Y255I, I257C, L287V, V288A	10.1
170	I257M, L286M, V288L	8.22
51 (Pecan)	F243M, S347G	8.03
188	P211A, N229T, S239T, G246A, F250A, L264M	7.96
183	S239T, G246A	6.14
191	N229T, S239T, G246A, L264V	5.79
109	Y255N, I257V, S314C	5.56

Figure S11. Identifying complementary luciferases for CouLuc-1 analogs via semi-rational library design. (a-c) CouLuc-1-NH_2 and (d-e) CouLuc-1-OH were screened against a panel of mutant luciferases using a protocol from Rathbun, *et al.*,[4] 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-NH_2 or (e) CouLuc-1-OH were classified as "hits" and their sequences were listed in (c) and (f), respectively.

a

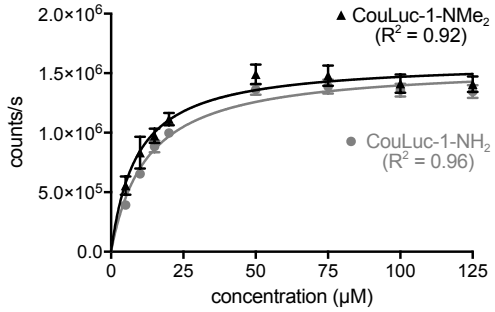


b

Luciferin	Luciferase	Photon output (p/s)	Fold improvement
CouLuc-1-NMe$_2$	Fluc	7.5 ± 1.5 x 10^7	77
	Pecan	5.8 ± 1.1 x 10^9	
CouLuc-1-NH$_2$	Fluc	2.3 ± 8.7 x 10^7	152
	Pecan	3.4 ± 0.56 x 10^9	
CouLuc-1-OH	Fluc	9.1 ± 1.7 x 10^6	77
	Pecan	7.0 ± 0.37 x 10^8	

Figure S12. 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.

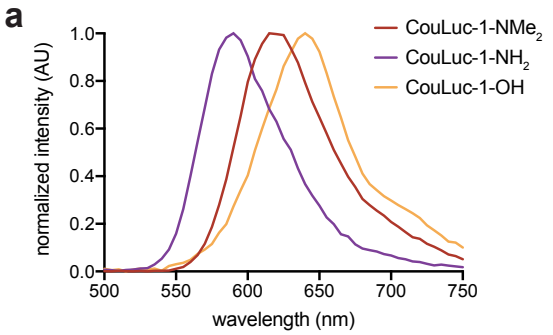
a



b

luciferin	luciferase	apparent K_M (μM)	Normalized k_{cat}/K_M[a]
D-luciferin	Fluc	20 ± 1.6	100 ± 12
CouLuc-1-NMe$_2$	Fluc	43 ± 4.1	1.1 ± 0.21 x 10^{-2}
	Pecan	8.9 ± 0.90	79 ± 11 x 10^{-2}
CouLuc-1-NH$_2$	Fluc	72 ± 7.7	2.9 ± 0.61 x 10^{-2}
	Pecan	12 ± 1.1	67 ± 10 x 10^{-2}
CouLuc-1-OH	Fluc	_[b]	_[b]
	Pecan	_[b]	_[b]

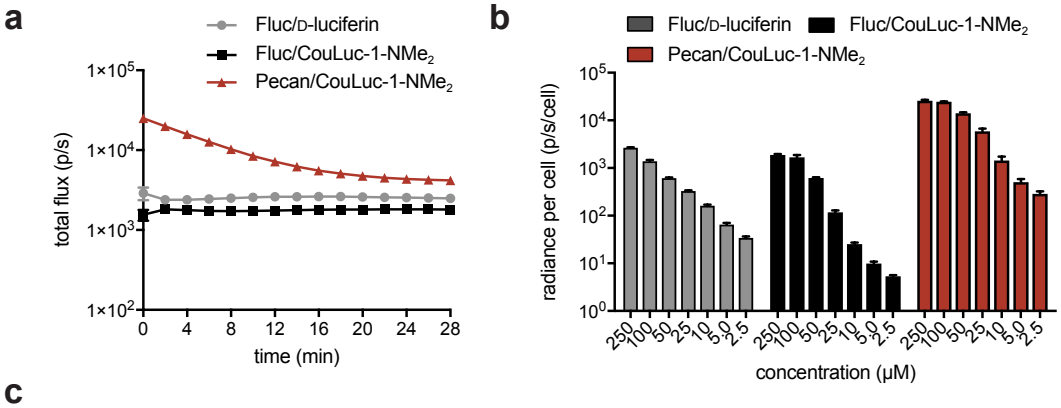
Figure S13. **Biochemical analyses of Fluc with CouLuc-1 analogs**. (a) Kinetics studies revealed CouLuc-1 analogs were poor binders of native luciferase. (b) Kinetic constants are apparent values, determined via measurements of initial light emission over a range of substrate concentrations. [a]Values 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.



b

Luciferin	Fluc $\lambda_{max,em}$	Pecan $\lambda_{max,em}$
D-luciferin	556	N.D.
CouLuc-1-NMe_2	620	625
CouLuc-1-NH_2	597	590
CouLuc-1-OH	625	640

Figure S14. Red-shifted bioluminescence was maintained with Pecan. (a) Recombinant Pecan was incubated with CouLuc-1 analogs and emission spectra were recorded. (b) Emission maxima (λ_{em}) for each analog. The corresponding emission maxima with Fluc are also shown.

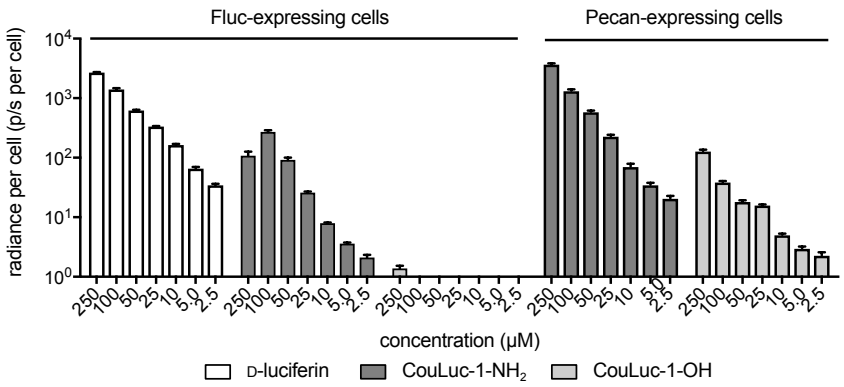


c

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-NMe_2	Fluc	4.6 ± 0.43 x 10^7	51 ± 0.40%	1.8 ± 0.17 x 10^3	69 ± 7.2%
CouLuc-1-NMe_2	**Pecan**	**5.0 ± 0.40 x 10^8**	**39 ± 0.72%**	**2.5 ± 0.21 x 10^4**	**960 ± 90%**

Figure S15. Cellular imaging with Pecan and CouLuc-1-NMe_2. HEK293 cells (5 x 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.

a



b

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-NH_2	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-NH_2	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 ± 0.13x 10^6	39 ± 0.72%	1.3 ± 0.71 x 10^2	5 ± 0.35%

Figure S16. 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-NH_2 (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.

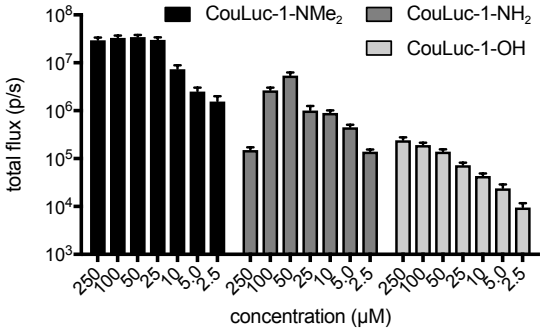
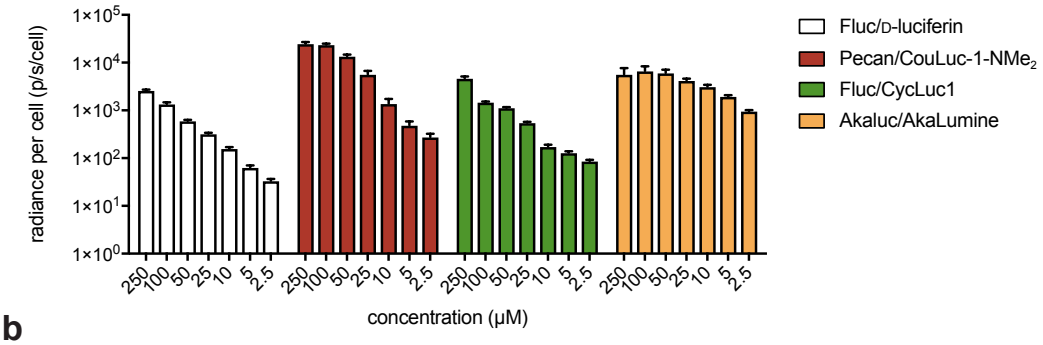


Figure S17. Robust light emission observed in DB7 cells. DB7 cells stably expressing Pecan (5 x 10^4) were incubated with CouLuc-1 analogs (250–2.5 μM). Photon outputs were measured immediately post substrate addition. Emission intensities are plotted as total photon flux values and error bars represent the standard error of the mean for n = 3 experiments.

a



b

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-NMe_2	Pecan	5.0 ± 0.40 x 10^8	39 ± 0.72%	2.5 ± 0.21 x 10^4	960 ± 90%
CycLuc1	Fluc	1.2 ± 0.10 x 10^8	51 ± 0.40%	4.7 ± 0.40 x 10^3	180 ± 18%
Akaluc	AkaLumine	5.7 ± 0.20 x 10^7	20 ± 0.49%	5.7 ± 0.20 x 10^3	218 ± 76%

Figure S18. **Cellular light emission from Pecan/CouLuc-1-NMe_2 is comparable to other red-emitting bioluminescence probes.** HEK293 cells (5 x 10^4) expressing mutant luciferases or Fluc were incubated with either D-luciferin (250–2.5 μM), CouLuc-1-NMe_2 (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.

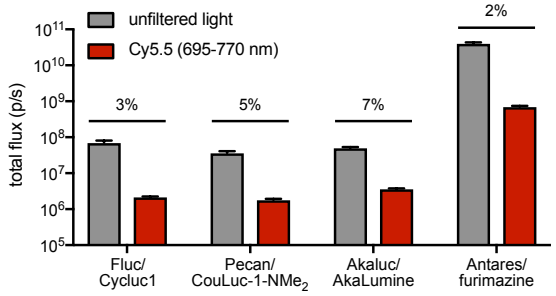


Figure S19. Pecan/CouLuc-1-NMe_2 produces a significant amount of near infrared photons. Luciferase-expressing DB7 cells (5 x 10^4) were treated with either CouLuc-1-NMe_2 (100 μM), D-luciferin (100 μM), CycLuc1 (100 μM), AkaLumine (100 μM) or furimazine (1:100 dilution from commercial stock). Photons 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.

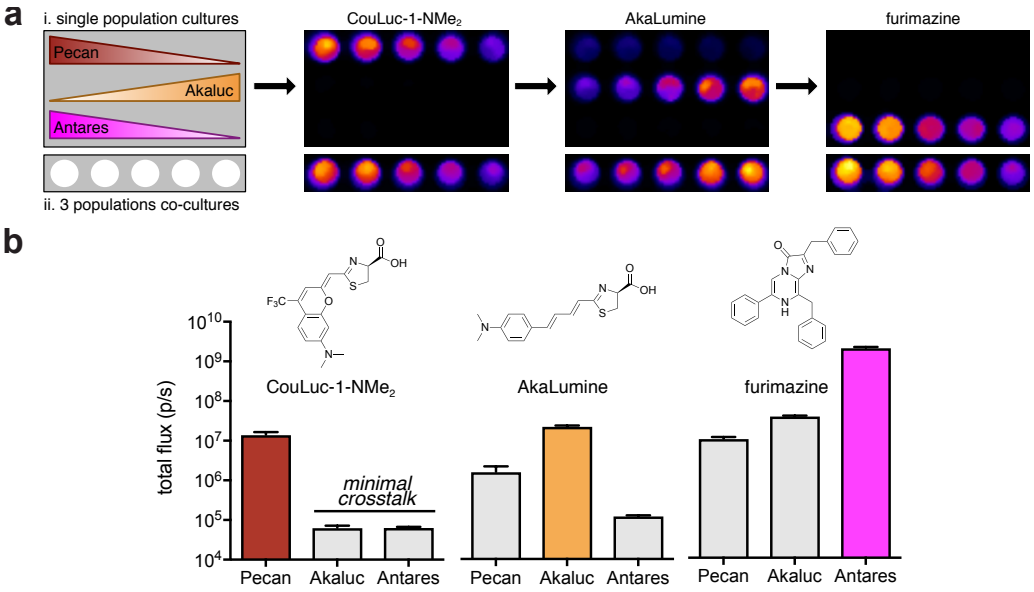


Figure S20. Multiplexed imaging with Pecan/CouLuc-1-NMe$_2$. Gradients of DB7 cells (1-4 x 10^4) expressing Pecan, Akaluc, or Antares were plated in a 96-well plate as shown. (a) Raw luminescent images from sequential substrate administration of CouLuc-1-NMe$_2$ (100 µM), AkaLumine (100 µM), and furimazine (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 10^4 luciferase-expressing cells were plotted. Minimal crosstalk was observed between Pecan/CouLuc-1-NMe$_2$ 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 $MgSO_4$, 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 10^6 DB7 cells stably expressing the luciferase of interest[5-6] 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 described.[5,7-8]

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

HEK293 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).[4-5] DB7 cells stably expressing Pecan, Akaluc, and Antares were generated according to Rathbun *et al.* via CRISPR-mediated gene insertion.[6] 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-IRES-eGFP or Pecan-IRES-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% CO_2 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 IRES-eGFP genes. Mutant luciferase inserts were amplified from pET vectors using the following primers:
5'- CGACTCACTATAGGGAGACCCAAGCTTATGGAAGATGCCAAAAACATTAAGAAG -3' and
5'-CACCGGCCTTATTCCAAGCGGCTTCGGCCAGTAACGTTTACACGGCGATCTTGCC-3'

IRES-eGFP insert was amplified from pcDNA vectors using the following primers:
5'- AAGGGCGGCAAGATCGCCGTGTAAACGTTACTGGCCGAAGCCGCTTGGAATAAG-3' and
5'-GCCGCCAGTGTGATGGATATCTGCAGAATTCttaCTTGTACAGCTCGTCCATGC-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 ºC 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.[9-10] The library template was first amplified using primers ZY040 and ZY041 (Table S2). 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 of mixture of mutagenic forward primers and ZY041 (Table S2). The reverse fragment reactions were performed using an equimolar mixture of mutagenic reverse primers and ZY040 (Table S2). 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 descried 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 *BamH*I and *Xba*I. 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 H_2O. 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 S2: Primers used to construct Rosetta CCM library. The bases highlighted in red denote sites targeted for mutagenesis.

Forward CCM Primers	
ZY040	ATCGCATCACCATCACCATCACGGATCCATGGAAGATGCCAAAAACATTAAGAAGG
RosCCM1-F-218	CGCTTGTGTCndtTTCAGTCATGCCC
RosCCM1-F-221	CGATTCAGTndtGCCCGCG
RosCCM1-F-222	GATTCAGTCATndtCGCGACCCCATC
RosCCM1-F-229	GACCCCATCTTCGGCndtCAGATCATC
RosCCM1-F-245	GCCATTTCACndtGGCTTCGGCAT
RosCCM1-F-246	CCATTTCACCACndtTTCGGCATGTT
RosCCM1-F-247	CACCACGGCndtGGCATGTTC
RosCCM1-F-250	CTTCGGCATGndtACCACGCTG
RosCCM1-F-251	CTTCGGCATGTTCndtACGCTGG
RosCCM1-F-254	TCACCACGCTGndtTACTTGATCTG
RosCCM1-F-314	GATCGCCndtGGCGGG
RosCCM1-F-338	GCATCCGCndtGGCTACGG
RosCCM1-F-342	AGGGCTACGGCndtACAGAAACAA
RosCCM1-F-343	CTACGGCCTGndtGAAACAACTAGTG
RosCCM1-F-347	CTGACAGAAACAACTndtGCCATTCTGATCACC
RosCCM1-F-351	TGCCATTCTGndtACCCCCGAAG
RosCCM1-F-352	CATTCTGATCndtCCCGAAGGGG
RosCCM1-F-420	GGCTGCACndtGGCGACATCGC
RosCCM1-F-437	TCATCGTGGACndtCTGAAGAGCC
RosCCM1-F-519	TGACCGGCndtTTGGACGCC

Reverse CCM Primers	
ZY041	TTTCGTTTTATTTGATGCCTCTAGATTACACGGCGATCTTGCCGCCCTTCTT
RosCCM1-R-218	GGGCATGACTGAAahnGACACAAGCG
RosCCM1-R-221	CGCGGGCahnACTGAATCG
RosCCM1-R-222	GATGGGGTCGCGahnATGACTGAATC
RosCCM1-R-229	GATGATCTGahnGCCGAAGATGGGGTC
RosCCM1-R-245	ATGCCGAAGCCahnGTGAAATGGC
RosCCM1-R-246	AACATGCCGAAahnGTGGTGAAATGG
RosCCM1-R-247	GAACATGCCahnGCCGTGGTG
RosCCM1-R-250	CAGCGTGGTahnCATGCCGAAG
RosCCM1-R-251	CCAGCGTahnGAACATGCCGAAG
RosCCM1-R-254	CAGATCAAGTAahnCAGCGTGGTGA
RosCCM1-R-314	CCCGCCahnGGCGATC
RosCCM1-R-338	CCGTAGCCahnGCGGATGC
RosCCM1-R-342	TTGTTTCTGTahnGCCGTAGCCCT
RosCCM1-R-343	CACTAGTTGTTTCahnCAGGCCGTAG
RosCCM1-R-347	GGTGATCAGAATGGCahnAGTTGTTTCTGTCAG
RosCCM1-R-351	CTTCGGGGGTahnCAGAATGGCA
RosCCM1-R-352	CCCCTTCGGGahnGATCAGAATG
RosCCM1-R-420	GCGATGTCGCCahnGTGCAGCC
RosCCM1-R-437	GGCTCTTCAGahnGTCCACGATGA
RosCCM1-R-519	GGCGTCCAAahnGCCGGTCA

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.[5] 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 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 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.*[4] 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[3], 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*.[6] 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*.[11] 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*.[5] 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*.[5] 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 μL. 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.[5] 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: 8442bff4fb7bf2ccb44655e8d15276c9bccfbbd0 using the ref15 score function.[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[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/[14] and subjected to an energy minimization using the UFF force field.[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- NMe_2 params file where LCC stands for CouLuc-1-NMe_2 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  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   0.20
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  S1   C2  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  S2   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  C4   O7  2
BOND_TYPE  N4   C9  1
BOND_TYPE  N4   C11 4
BOND_TYPE  N4   C14 4
BOND_TYPE  O4   C6  1
BOND_TYPE  O4   C9  1
BOND_TYPE  C5   C6  1
BOND_TYPE  C5   H4  1
BOND_TYPE  C5   H5  1
BOND_TYPE  N5   C12 4
BOND_TYPE  N5   C14 4
BOND_TYPE  N5   H2  1
BOND_TYPE  O5   C7  1
BOND_TYPE  O5   H3  1
BOND_TYPE  C6   C7  1
BOND_TYPE  C6   H9  1
BOND_TYPE  N6   C13 1
BOND_TYPE  N6   H6  1
BOND_TYPE  N6   H7  1
BOND_TYPE  O6   C8  1
BOND_TYPE  O6   H8  1
BOND_TYPE  C7   C8  1
BOND_TYPE  C7   H11 1
BOND_TYPE  N7   H10 1
BOND_TYPE  C8   C9  1
BOND_TYPE  C8   H12 1
BOND_TYPE  N8   C26 1
BOND_TYPE  N8   C27 1
BOND_TYPE  O8   C18 4
BOND_TYPE  C9   H13 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
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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  H24 1
BOND_TYPE  C27  H25 1
BOND_TYPE  C27  H26 1
CHI 1  C8   C7   O5   H3
PROTON_CHI 1 SAMPLES 21 45 50 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 50 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   C3   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
CHI 13  C23  C22  N8   C26
NBR_ATOM  N7
NBR_RADIUS 16.480287
ICOOR_INTERNAL    N7     0.000000    0.000000    0.000000   N7    S2    O1
ICOOR_INTERNAL    S2     0.000000  180.000000    1.650062   N7    S2    O1
ICOOR_INTERNAL    O1     0.000000   72.104658    1.437728   S2    N7    O1
ICOOR_INTERNAL    O2  -110.996100   68.074449    1.445125   S2    N7    O1
ICOOR_INTERNAL    O3  -129.794407   67.944568    1.509427   S2    N7    O2
ICOOR_INTERNAL    C5    48.026210   59.837306    1.410874   O3    S2    N7
ICOOR_INTERNAL    C6   157.652992   70.710750    1.511242   C5    O3    S2
ICOOR_INTERNAL    O4    77.282034   69.984387    1.402847   C6    C5    O3
ICOOR_INTERNAL    C9   125.140105   74.095561    1.413122   O4    C6    C5
ICOOR_INTERNAL    N4  -109.511147   70.744428    1.445332   C9    O4    C6
ICOOR_INTERNAL    C11 -150.220766   54.143952    1.365802   N4    C9    O4
ICOOR_INTERNAL    N3    -0.899108   46.184845    1.342623   C11   N4    C9
ICOOR_INTERNAL    C10 -179.435301   59.581414    1.325031   N3    C11   N4
ICOOR_INTERNAL    N2    -0.833691   57.581285    1.332176   C10   N3    C11
ICOOR_INTERNAL    C13    0.541646   58.153548    1.342096   N2    C10   N3
ICOOR_INTERNAL    N6  -179.885440   60.506917    1.449335   C13   N2    C10
ICOOR_INTERNAL    H6    -0.115371   59.977458    0.984393   N6    C13   N2
ICOOR_INTERNAL    H7   179.945780   60.016656    0.985154   N6    C13   H6
ICOOR_INTERNAL    C12  179.862428   62.184716    1.420651   C13   N2    N6
ICOOR_INTERNAL    N5  -179.949517   44.621863    1.328601   C12   C13   N2
ICOOR_INTERNAL    C14  179.677558   70.203038    1.324715   N5    C12   C13
ICOOR_INTERNAL    H15 -179.578659   55.002841    1.032967   C14   N5    C12
ICOOR_INTERNAL    H2  -179.970617   54.849045    0.984976   N5    C12   C14
ICOOR_INTERNAL    H14  179.967987   61.153361    1.031699   C10   N3    N2
ICOOR_INTERNAL    C8   125.386647   71.678423    1.475682   C9    O4    N4
ICOOR_INTERNAL    O6    90.159142   71.814346    1.374530   C8    C9    O4
ICOOR_INTERNAL    H8  -179.907404   70.535671    0.969463   O6    C8    C9
ICOOR_INTERNAL    C7  -120.342024   77.546124    1.466197   C8    C9    O6
ICOOR_INTERNAL    O5   150.569759   65.740398    1.379514   C7    C8    C9
ICOOR_INTERNAL    H3   179.991726   70.530485    0.970413   O5    C7    C8
ICOOR_INTERNAL    H11  117.079816   69.012841    1.070864   C7    C8    O5
ICOOR_INTERNAL    H12 -121.174077   63.727852    1.069712   C8    C9    C7
```

```
ICOOR_INTERNAL    H13  117.670342   67.676021    1.069572   C9    O4    C8
ICOOR_INTERNAL    H9  -119.425559   70.515230    1.070763   C6    C5    O4
ICOOR_INTERNAL    H4  -120.035866   70.426804    1.069592   C5    O3    C6
ICOOR_INTERNAL    H5  -119.906355   70.417410    1.069976   C5    O3    H4
ICOOR_INTERNAL    C4   -83.793754   58.281421    1.467467   N7    S2    O1
ICOOR_INTERNAL    C3   178.602413   63.790977    1.496597   C4    N7    S2
ICOOR_INTERNAL    N1   171.710317   59.685217    1.308129   C3    C4    N7
ICOOR_INTERNAL    C1   179.403075   66.538537    1.363242   N1    C3    C4
ICOOR_INTERNAL    C15  178.038432   54.751568    1.475845   C1    N1    C3
ICOOR_INTERNAL    C16 -179.973359   56.742589    1.347238   C15   C1    N1
ICOOR_INTERNAL    O8     1.725866   59.229554    1.354592   C16   C15   C1
ICOOR_INTERNAL    C18  179.858798   56.623479    1.354364   O8    C16   C15
ICOOR_INTERNAL    C19    0.887588   59.212928    1.412822   C18   O8    C16
ICOOR_INTERNAL    C20   -0.844484   61.931272    1.515819   C19   C18   O8
ICOOR_INTERNAL    C17    0.091368   61.915940    1.352930   C20   C19   C18
ICOOR_INTERNAL    H17 -179.883717   59.714914    1.087586   C17   C20   C19
ICOOR_INTERNAL    C25 -179.669554   53.469135    1.526954   C20   C19   C17
ICOOR_INTERNAL    F1    -0.147422   65.088860    1.374607   C25   C20   C19
ICOOR_INTERNAL    F2  -120.743410   70.959525    1.384707   C25   C20   F1
ICOOR_INTERNAL    F3  -118.465114   70.887590    1.383661   C25   C20   F2
ICOOR_INTERNAL    C24 -179.813659   62.667377    1.407529   C19   C18   C20
ICOOR_INTERNAL    C23    0.444229   58.666119    1.396868   C24   C19   C18
ICOOR_INTERNAL    C22    0.207422   58.151002    1.413573   C23   C24   C19
ICOOR_INTERNAL    C21   -0.508039   63.430140    1.413587   C22   C23   C24
ICOOR_INTERNAL    H20  179.170241   57.531909    1.075128   C21   C22   C23
ICOOR_INTERNAL    N8  -179.834487   58.360800    1.454020   C22   C23   C21
ICOOR_INTERNAL    C26  -15.367154   57.151155    1.459721   N8    C22   C23
ICOOR_INTERNAL    H21   -4.544141   65.075936    1.099691   C26   N8    C22
ICOOR_INTERNAL    H22 -118.257943   70.626381    1.111531   C26   N8    H21
ICOOR_INTERNAL    H23 -119.101486   70.203809    1.111081   C26   N8    H22
ICOOR_INTERNAL    C27 -179.704739   56.873654    1.458277   N8    C22   C26
ICOOR_INTERNAL    H24   -3.336325   65.036189    1.098860   C27   N8    C22
ICOOR_INTERNAL    H25 -118.846485   70.514855    1.110651   C27   N8    H24
ICOOR_INTERNAL    H26 -119.305614   70.093290    1.110606   C27   N8    H25
ICOOR_INTERNAL    H19 -179.115566   64.471844    1.076073   C23   C24   C22
ICOOR_INTERNAL    H18 -179.865470   56.842756    1.072154   C24   C19   C23
ICOOR_INTERNAL    H16 -179.288691   62.704338    1.086883   C15   C1    C16
ICOOR_INTERNAL    S1  -178.645915   71.214035    1.654766   C1    N1    C15
ICOOR_INTERNAL    C2     0.064084   84.137315    1.751848   S1    C1    N1
ICOOR_INTERNAL    H1  -179.571667   51.154423    1.031194   C2    S1    C1
ICOOR_INTERNAL    O7   179.926420   56.571828    1.227619   C4    N7    C3
ICOOR_INTERNAL    H10  179.925774   60.889832    0.984604   N7    S2    C4
```

The contents of the CouLuc-1-NH_2 ligand params file where LCD stands for CouLuc-1-NH_2 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
```

```
ATOM  N6  NH2O  X   -0.37
ATOM  H6  Hpol  X   0.53
ATOM  H7  Hpol  X   0.53
ATOM  C12 aroC  X   -0.01
ATOM  N5  Ntrp  X   -0.51
ATOM  C14 aroC  X   -0.01
ATOM  H15 Haro  X   0.22
ATOM  H2  Hpol  X   0.53
ATOM  H14 Haro  X   0.22
ATOM  C8  CH1   X   0.01
ATOM  O6  OH    X   -0.56
ATOM  H8  Hpol  X   0.53
ATOM  C7  CH1   X   0.01
ATOM  O5  OH    X   -0.56
ATOM  H3  Hpol  X   0.53
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   0.72
ATOM  C3  aroC  X   -0.01
ATOM  N1  Nhis  X   -0.43
ATOM  C1  aroC  X   -0.01
ATOM  C15 CH1   X   0.01
ATOM  C16 COO   X   0.72
ATOM  O8  OOC   X   -0.66
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   0.01
ATOM  F1  F     X   -0.15
ATOM  F2  F     X   -0.15
ATOM  F3  F     X   -0.15
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  NH2O  X   -0.37
ATOM  H21 Hpol  X   0.53
ATOM  H22 Hpol  X   0.53
ATOM  H19 Haro  X   0.22
ATOM  H18 Haro  X   0.22
ATOM  H16 Hapo  X   0.20
ATOM  S1  S     X   -0.06
ATOM  C2  aroC  X   -0.01
ATOM  H1  Haro  X   0.22
ATOM  O7  ONH2  X   -0.45
ATOM  H10 Hpol  X   0.53
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  S1   C2  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  S2   O3  1
BOND_TYPE  S2   N7  1
```

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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  C4   O7  2
BOND_TYPE  N4   C9  1
BOND_TYPE  N4   C11 4
BOND_TYPE  N4   C14 4
BOND_TYPE  O4   C6  1
BOND_TYPE  O4   C9  1
BOND_TYPE  C5   C6  1
BOND_TYPE  C5   H4  1
BOND_TYPE  C5   H5  1
BOND_TYPE  N5   C12 4
BOND_TYPE  N5   C14 4
BOND_TYPE  N5   H2  1
BOND_TYPE  O5   C7  1
BOND_TYPE  O5   H3  1
BOND_TYPE  C6   C7  1
BOND_TYPE  C6   H9  1
BOND_TYPE  N6   C13 1
BOND_TYPE  N6   H6  1
BOND_TYPE  N6   H7  1
BOND_TYPE  O6   C8  1
BOND_TYPE  O6   H8  1
BOND_TYPE  C7   C8  1
BOND_TYPE  C7   H11 1
BOND_TYPE  N7   H10 1
BOND_TYPE  C8   C9  1
BOND_TYPE  C8   H12 1
BOND_TYPE  N8   H21 1
BOND_TYPE  N8   H22 1
BOND_TYPE  O8   C18 4
BOND_TYPE  C9   H13 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  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
CHI 1  C8   C7   O5   H3
PROTON_CHI 1 SAMPLES 21 45 50 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 50 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
```

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CHI 4  N7   S2   O3   C5
CHI 5  C4   N7   S2   O1
CHI 6  N7   C4   C3   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    1.649480   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    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   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    C12  179.862428   62.184716    1.420651   C13   N2    N6
ICOOR_INTERNAL    N5  -179.949517   44.621863    1.328601   C12   C13   N2
ICOOR_INTERNAL    C14  179.677558   70.203038    1.324715   N5    C12   C13
ICOOR_INTERNAL    H15 -179.517871   55.041066    1.032119   C14   N5    C12
ICOOR_INTERNAL    H2  -179.976464   54.922095    0.984330   N5    C12   C14
ICOOR_INTERNAL    H14 -179.959018   61.152494    1.031712   C10   N3    N2
ICOOR_INTERNAL    C8   125.404314   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  -120.391196   77.527376    1.465575   C8    C9    O6
ICOOR_INTERNAL    O5   150.567803   65.743236    1.380198   C7    C8    C9
ICOOR_INTERNAL    H3   179.981880   70.545702    0.969832   O5    C7    C8
ICOOR_INTERNAL    H11  117.168004   68.989317    1.070413   C7    C8    O5
ICOOR_INTERNAL    H12 -121.158644   63.838599    1.069836   C8    C9    C7
ICOOR_INTERNAL    H13  117.674596   67.713631    1.070064   C9    O4    C8
ICOOR_INTERNAL    H9  -119.374097   70.454273    1.069969   C6    C5    O4
ICOOR_INTERNAL    H4  -119.990464   70.450854    1.069592   C5    O3    C6
ICOOR_INTERNAL    H5  -119.938832   70.434132    1.070190   C5    O3    H4
ICOOR_INTERNAL    C4   -83.828030   58.301695    1.468382   N7    S2    O1
ICOOR_INTERNAL    C3   178.603415   63.816343    1.496095   C4    N7    S2
ICOOR_INTERNAL    N1   171.714611   59.715820    1.309092   C3    C4    N7
ICOOR_INTERNAL    C1   179.404452   66.574660    1.362844   N1    C3    C4
ICOOR_INTERNAL    C15  179.343162   54.627824    1.477523   C1    N1    C3
ICOOR_INTERNAL    C16  179.978262   56.646772    1.345610   C15   C1    N1
ICOOR_INTERNAL    O8     0.981830   59.223614    1.354162   C16   C15   C1
ICOOR_INTERNAL    C18  179.745138   56.639084    1.351881   O8    C16   C15
ICOOR_INTERNAL    C19    0.484766   59.068241    1.419987   C18   O8    C16
ICOOR_INTERNAL    C20   -0.558169   62.167587    1.515074   C19   C18   O8
ICOOR_INTERNAL    C17    0.167837   61.905154    1.351574   C20   C19   C18
ICOOR_INTERNAL    H17 -179.921795   59.732081    1.088256   C17   C20   C19
ICOOR_INTERNAL    C25 -179.891720   53.582674    1.526174   C20   C19   C17
ICOOR_INTERNAL    F1    -0.070420   65.114755    1.374596   C25   C20   C19
ICOOR_INTERNAL    F2  -120.759332   70.951926    1.385320   C25   C20   F1
ICOOR_INTERNAL    F3  -118.384368   70.903342    1.383675   C25   C20   F2
ICOOR_INTERNAL    C24 -179.946722   62.419046    1.414589   C19   C18   C20
ICOOR_INTERNAL    C23    0.010362   59.023358    1.398205   C24   C19   C18
ICOOR_INTERNAL    C22    0.193614   59.392855    1.396772   C23   C24   C19
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ICOOR_INTERNAL    C21   -0.022927   60.637687    1.395543   C22   C23   C24
ICOOR_INTERNAL    H20  179.999029   60.022004    1.082887   C21   C22   C23
ICOOR_INTERNAL    N8  -179.722109   59.603023    1.416893   C22   C23   C21
ICOOR_INTERNAL    H21  179.543056   59.258297    1.030065   N8    C22   C23
ICOOR_INTERNAL    H22 -179.490041   59.252862    1.031163   N8    C22   H21
ICOOR_INTERNAL    H19 -179.986643   60.635697    1.083038   C23   C24   C22
ICOOR_INTERNAL    H18 -179.867044   56.526093    1.071263   C24   C19   C23
ICOOR_INTERNAL    H16 -179.940476   62.786240    1.087599   C15   C1    C16
ICOOR_INTERNAL    S1  -179.929034   71.177075    1.654766   C1    N1    C15
ICOOR_INTERNAL    C2     0.061092   84.158025    1.751119   S1    C1    N1
ICOOR_INTERNAL    H1  -179.547396   51.165815    1.032145   C2    S1    C1
ICOOR_INTERNAL    O7   179.981870   56.614562    1.227206   C4    N7    C3
ICOOR_INTERNAL    H10  179.984530   60.814997    0.984262   N7    S2    C4
```

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  C8  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
```

```
ATOM  C18 aroC  X   -0.00
ATOM  C19 aroC  X   -0.00
ATOM  C20 aroC  X   -0.00
ATOM  C17 aroC  X   -0.00
ATOM  H17 Haro  X   0.23
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   -0.00
ATOM  C23 aroC  X   -0.00
ATOM  C22 aroC  X   -0.00
ATOM  C21 aroC  X   -0.00
ATOM  H20 Haro  X   0.23
ATOM  O9  OH    X   -0.55
ATOM  H21 Hpol  X   0.54
ATOM  H19 Haro  X   0.23
ATOM  H18 Haro  X   0.23
ATOM  H16 Hapo  X   0.21
ATOM  S1  S     X   -0.05
ATOM  C2  aroC  X   -0.00
ATOM  H1  Haro  X   0.23
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  S1   C2  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  S2   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  C4   O7  2
BOND_TYPE  N4   C9  1
BOND_TYPE  N4   C11 4
BOND_TYPE  N4   C14 4
BOND_TYPE  O4   C6  1
BOND_TYPE  O4   C9  1
BOND_TYPE  C5   C6  1
BOND_TYPE  C5   H4  1
BOND_TYPE  C5   H5  1
BOND_TYPE  N5   C12 4
BOND_TYPE  N5   C14 4
BOND_TYPE  N5   H2  1
BOND_TYPE  O5   C7  1
BOND_TYPE  O5   H3  1
BOND_TYPE  C6   C7  1
BOND_TYPE  C6   H9  1
BOND_TYPE  N6   C13 1
BOND_TYPE  N6   H6  1
BOND_TYPE  N6   H7  1
BOND_TYPE  O6   C8  1
BOND_TYPE  O6   H8  1
BOND_TYPE  C7   C8  1
BOND_TYPE  C7   H11 1
BOND_TYPE  N7   H10 1
```

```
BOND_TYPE  C8   C9  1
BOND_TYPE  C8   H12 1
BOND_TYPE  O8   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  H18 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 50 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 50 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   S2    N7    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   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    C12  179.862428   62.184716    1.420651   C13   N2    N6
ICOOR_INTERNAL    N5  -179.949517   44.621863    1.328601   C12   C13   N2
ICOOR_INTERNAL    C14  179.677558   70.203038    1.324715   N5    C12   C13
ICOOR_INTERNAL    H15 -179.517871   55.041066    1.032119   C14   N5    C12
ICOOR_INTERNAL    H2  -179.976464   54.922095    0.984330   N5    C12   C14
ICOOR_INTERNAL    H14 -179.959018   61.152494    1.031712   C10   N3    N2
ICOOR_INTERNAL    C8   125.404314   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  -120.391196   77.527376    1.465575   C8    C9    O6
ICOOR_INTERNAL    O5   150.567803   65.743236    1.380198   C7    C8    C9
ICOOR_INTERNAL    H3   179.981880   70.545702    0.969832   O5    C7    C8
ICOOR_INTERNAL    H11  117.168004   68.989317    1.070413   C7    C8    O5
ICOOR_INTERNAL    H12 -121.158644   63.838599    1.069836   C8    C9    C7
ICOOR_INTERNAL    H13  117.674596   67.713631    1.070064   C9    O4    C8
ICOOR_INTERNAL    H9  -119.374097   70.454273    1.069969   C6    C5    O4
ICOOR_INTERNAL    H4  -119.990464   70.450854    1.069592   C5    O3    C6
ICOOR_INTERNAL    H5  -119.938832   70.434132    1.070190   C5    O3    H4
ICOOR_INTERNAL    C4   -83.828030   58.301695    1.468382   N7    S2    O1
ICOOR_INTERNAL    C3   178.603415   63.816343    1.496095   C4    N7    S2
ICOOR_INTERNAL    N1   171.714611   59.715820    1.309092   C3    C4    N7
ICOOR_INTERNAL    C1   179.404452   66.574660    1.362844   N1    C3    C4
ICOOR_INTERNAL    C15  179.223421   54.664545    1.476648   C1    N1    C3
ICOOR_INTERNAL    C16 -179.989679   56.710153    1.346762   C15   C1    N1
ICOOR_INTERNAL    O8     0.912521   59.265165    1.353938   C16   C15   C1
ICOOR_INTERNAL    C18  179.613843   56.700286    1.353045   O8    C16   C15
ICOOR_INTERNAL    C19    0.696119   58.989698    1.419737   C18   O8    C16
ICOOR_INTERNAL    C20   -0.534929   62.229493    1.516594   C19   C18   O8
ICOOR_INTERNAL    C17   -0.014746   61.919117    1.352467   C20   C19   C18
ICOOR_INTERNAL    H17 -179.863883   59.795042    1.088156   C17   C20   C19
ICOOR_INTERNAL    C25 -179.760539   53.572574    1.525937   C20   C19   C17
ICOOR_INTERNAL    F1    -0.152786   65.152910    1.374488   C25   C20   C19
ICOOR_INTERNAL    F2  -120.702945   70.924488    1.383259   C25   C20   F1
ICOOR_INTERNAL    F3  -118.504269   70.882734    1.383434   C25   C20   F2
ICOOR_INTERNAL    C24 -179.969607   62.368500    1.414553   C19   C18   C20
ICOOR_INTERNAL    C23    0.083722   59.067642    1.398551   C24   C19   C18
ICOOR_INTERNAL    C22    0.123564   59.352531    1.393135   C23   C24   C19
ICOOR_INTERNAL    C21   -0.006879   60.580877    1.397258   C22   C23   C24
ICOOR_INTERNAL    H20  179.995074   60.167720    1.083846   C21   C22   C23
ICOOR_INTERNAL    O9  -179.830419   60.331140    1.346965   C22   C23   C21
ICOOR_INTERNAL    H21  179.547800   57.851533    0.967795   O9    C22   C23
ICOOR_INTERNAL    H19 -179.995294   60.475787    1.083563   C23   C24   C22
ICOOR_INTERNAL    H18  179.968998   56.552726    1.071404   C24   C19   C23
ICOOR_INTERNAL    H16 -179.899585   62.673003    1.087086   C15   C1    C16
ICOOR_INTERNAL    S1  -179.809293   71.177075    1.654766   C1    N1    C15
ICOOR_INTERNAL    C2     0.061092   84.158025    1.751119   S1    C1    N1
ICOOR_INTERNAL    H1  -179.547396   51.165815    1.032145   C2    S1    C1
ICOOR_INTERNAL    O7   179.981870   56.614562    1.227206   C4    N7    C3
ICOOR_INTERNAL    H10  179.984530   60.814997    0.984262   N7    S2    C4
```

The Fluc structure was used as input to the RosettaMatch protocol.[16] 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.

```
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
 -ex1aro
 -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:euclid_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_matchres_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:

```
<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
    -linmem_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:

```
<ROSETTASCRIPTS>

       <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"/>
              <AddOrRemoveMatchCsts name="addprg" cst_instruction=add_pregenerated/>
              #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"/>
```

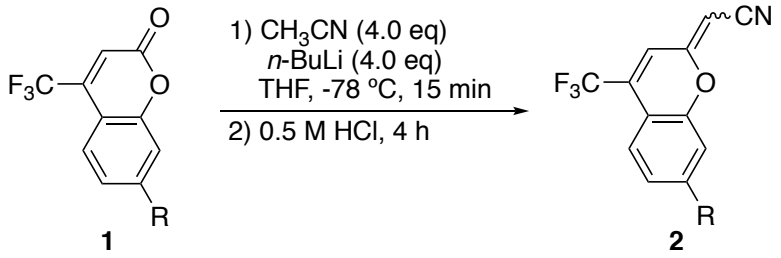
```
            #Minimize after each design.
            <EnzRepackMinimize name="min" scorefxn_minimize="ref2015" cst_opt="0"
design="0" repack_only="0" fix_catalytic="1" minimize_rb="1" minimize_bb="1"
minimize_sc="1" minimize_lig="1" min_in_stages="1" backrub="0" cycles="1"
task_operations="dsgn_cuts_off"/>

            #Perform a final repacking step.
            <EnzRepackMinimize name="rpkmin" scorefxn_minimize="ref2015" cst_opt="0"
design="0" repack_only="1" fix_catalytic="0" minimize_rb="1" minimize_bb="1"
minimize_sc="1" minimize_lig="0" min_in_stages="1" backrub="0" cycles="1"
task_operations="dsgn_cuts_off"/>

            #Monte Carlo movers for each step in the enzdes process (helps to
generate )
            <GenericMonteCarlo name="multi_cstopt" mover_name="cstopt"
scorefxn_name="ref2015" trials="10" sample_type="low" temperature="0.6" drift="1"
recover_low="1" preapply="0"/>
      </MOVERS>

      <PROTOCOLS>
            <Add mover_name="addcst"/>
            <Add mover_name="multi_cstopt"/>
            <Add mover_name="dsgn"/>
            <Add mover_name="min"/>
            <Add mover_name="dsgn"/>
            <Add mover_name="min"/>
            <Add mover_name="dsgn"/>
            <Add mover_name="min"/>
            Add mover=rmvcst/>
            Add mover=rpkmin/>
            Add mover=des_min/>
            Add mover=des_min/>
            Add mover=des_min/>
            Add mover_name="rmvcst"/>
            Add mover_name="rpkmin"/>
            Add mover=finmin_rpkmin/>
      </PROTOCOLS>

</ROSETTASCRIPTS>
```

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. ^{1}H NMR and ^{13}C 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 ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. Data for ^{13}C 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 spectrofluorometer operated by FelixGX 4.2.2 software, with 5 nm excitation and emission slit

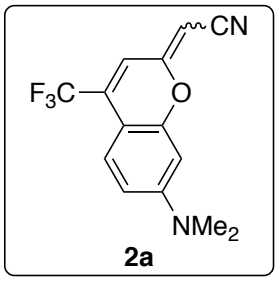
widths, 0.1 s integration rate, and enabled emission correction. Data analysis and curve fitting were performed using MS Excel 2019 and GraphPad Prism 8.

Synthetic procedures



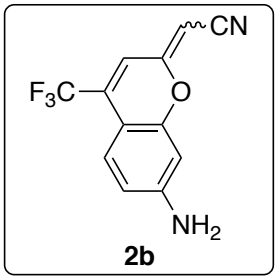
General procedure for the synthesis of nitrile (2)

To a solution of CH_3CN (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 °C 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_4Cl 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_2SO_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).

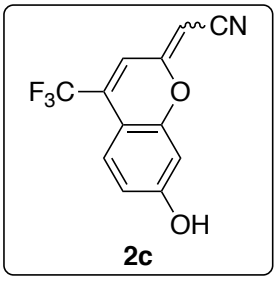
Following the general procedure using commercial 7-(dimethylamino)-4-(trifluoromethyl)coumarin (**1a**) (514 mg, 2.0 mmol). Purification by flash chromatography on silica gel (hexanes/EtOAc, 0% to 20%) afforded **2a** as an orange solid (358 mg, 64% yield). ^{1}H NMR ($CDCl_3$, 400 MHz, compound exists 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 $C_{14}H_{12}F_3N_2O$ $(M+H)^+$ 290.0896, observed 290.0900; *E*-isomer $C_{14}H_{12}F_3N_2O$ $(M+H)^+$ 290.0896, observed 290.0901.



2b

(Z/E)-2-(7-Amino-4-(trifluoromethyl)-2H-chromen-2-ylidene)acetonitrile (2b).

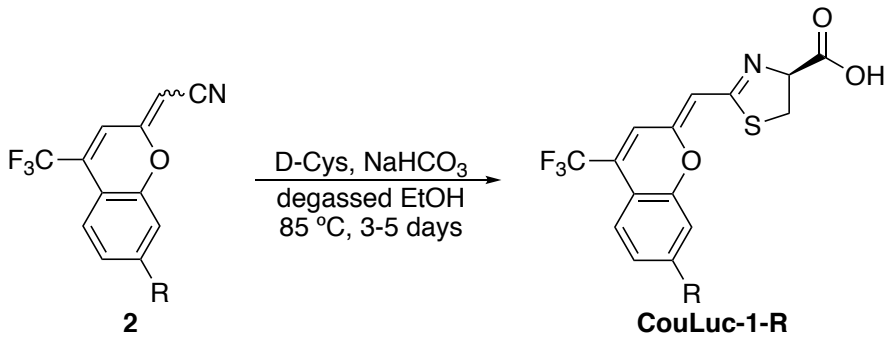
Following the general procedure using commercial 7-amino-4-(trifluoromethyl)coumarin (**1b**) (458 mg, 2.0 mmol). Purification by flash chromatography on silica gel (hexanes/EtOAc, 0% to 30%) afforded **2b** as an orange solid (308 mg, 61% yield). ^{1}H NMR (CD_3CN, 400 MHz, compound exists as a mixture 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*); ^{13}C NMR (CD_3CN, 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*; ^{19}F NMR (CD_3CN, 377 MHz) δ -64.6*, -64.7§; HRMS (ESI) calculated for $C_{12}H_8F_3N_2O$ $(M+H)^+$ 253.0583, observed 253.0582.



2c

(Z/E)-2-(7-Hydroxy-4-(trifluoromethyl)-2H-chromen-2-ylidene)acetonitrile (2c).

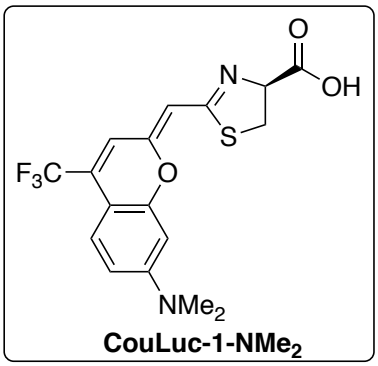
Following the general procedure using commercial 7-hydroxy-4-(trifluoromethyl)coumarin (**1c**) (460 mg, 2.0 mmol). Purification by flash chromatography on silica gel (hexanes/EtOAc, 0% to 50%) afforded **2c** as a yellow solid (354 mg, 70% yield). ^{1}H NMR (CD_3OD, 400 MHz, compound exists as a mixture 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*); ^{13}C NMR (CD_3OD, 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*; ^{19}F NMR (CD_3OD, 377 MHz) δ -66.1§, -66.2*; HRMS (ESI) calculated for $C_{12}H_5F_3NO_2$ $(M–H)^–$ 252.0278, observed 252.0270.



2 **CouLuc-1-R**

General procedure for the synthesis of CouLuc-1-R

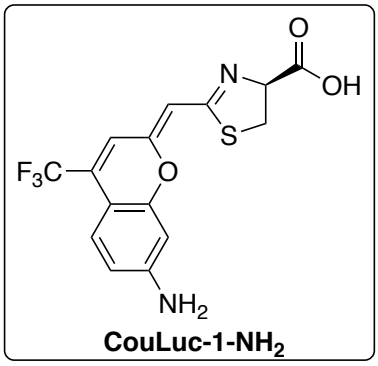
To a microwave vial containing nitrile (**2**) (0.15 mmol, 1.0 eq), D-cysteine hydrochloride monohydrate (0.23 mmol, 1.5 eq) and $NaHCO_3$ (0.60 mmol, 4.0 eq) was added N_2-sparged EtOH (1.5 mL). The suspension was heated at 85 ºC under N_2 and monitored by LC/MS. After 3-5 days the consumption of **2** 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_2O (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**.



CouLuc-1-NMe$_2$

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

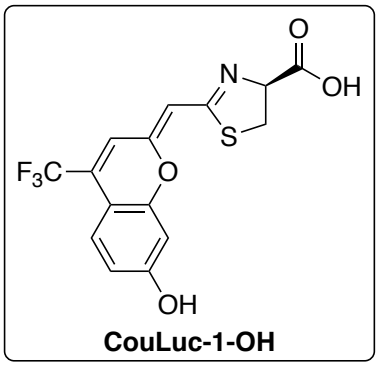
Following the general procedure using **2a** (42 mg, 0.15 mmol), **CouLuc-1-NMe$_2$** was obtained as a red solid (23 mg, 40% yield). ^{1}H NMR (CD_3OD + TFA-d_1, 500 MHz) δ 7.56 – 7.53 (m, 1H), 6.93 (s, 1H), 6.89 (dd, J = 9.3, 2.6 Hz, 1H), 6.76 (d, J = 2.6 Hz, 1H), 6.18 (s, 1H), 5.16 (dd, J = 9.4, 5.6 Hz, 1H), 4.00 – 3.90 (m, 2H), 3.14 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6 + TFA-d_1) δ 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) δ -63.4; HRMS (ESI) calculated for $C_{17}H_{15}F_3N_2O_3S$ $(M+H)^+$ 385.0828, observed 385.0833.



CouLuc-1-NH$_2$

(Z)-2-((7-Amino-4-(trifluoromethyl)-2H-chromen-2-ylidene)methyl)-4,5-dihydro-thiazole-4-carboxylic acid (CouLuc-1-NH$_2$).

Following the general procedure using **2b** (37 mg, 0.15 mmol), **CouLuc-1-NH$_2$** was obtained as an orange solid (28 mg, 52% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.09 – 7.02 (m, 1H), 6.87 (s, 1H), 6.45 – 6.38 (m, 2H), 6.14 (s, 2H), 5.94 (s, 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); ^{13}C NMR (125 MHz, DMSO-d_6) 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; ^{19}F NMR (DMSO-d_6, 377 MHz) δ -63.4; HRMS (ESI) calculated for $C_{15}H_{12}F_3N_2O_3S$ $(M+H)^+$ 357.0515, observed 357.0523.

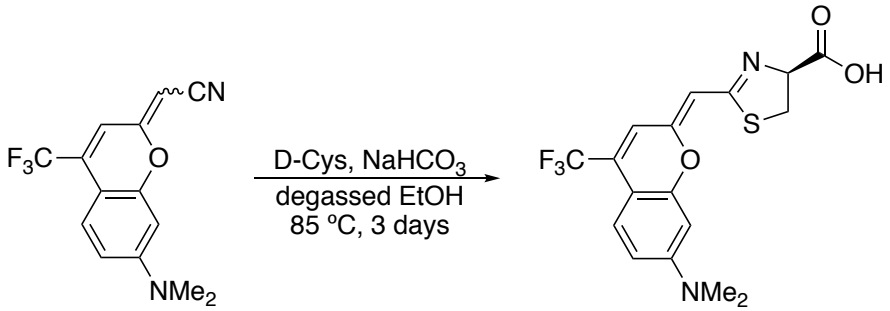


CouLuc-1-OH

(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). ^{1}H NMR (500 MHz, CD_3OD + TFA-d_1) 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); ^{13}C NMR (125 MHz, CD_3OD + TFA-d_1) 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; ^{19}F NMR (CD_3OD + TFA-d_1, 377 MHz) δ -65.5; HRMS (ESI) calculated for $C_{15}H_{11}F_3NO_4S$ $(M+H)^+$ 358.0355, observed 358.0358.

Synthesis of CouLuc-1-NMe$_2$ with chromatography-free procedure



2a **CouLuc-1-NMe$_2$**

To a reaction flask containing nitrile (**2a**) (1.25 g, 4.49 mmol), D-cysteine hydrochloride monohydrate (1.18 g, 6.73 mmol) and $NaHCO_3$ (1.51 g, 17.94 mmol) was N_2-sparged EtOH (45 mL) was heated at 85 ºC under N_2. After heating for 3 days the EtOH was evaporated under vacuum. The yellow solid was triturated with Et_2O (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-NMe$_2$** as a red solid (468 mg, 27% yield) to provide high purity material by NMR (>95%).

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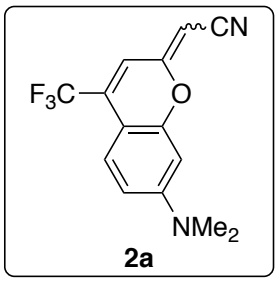
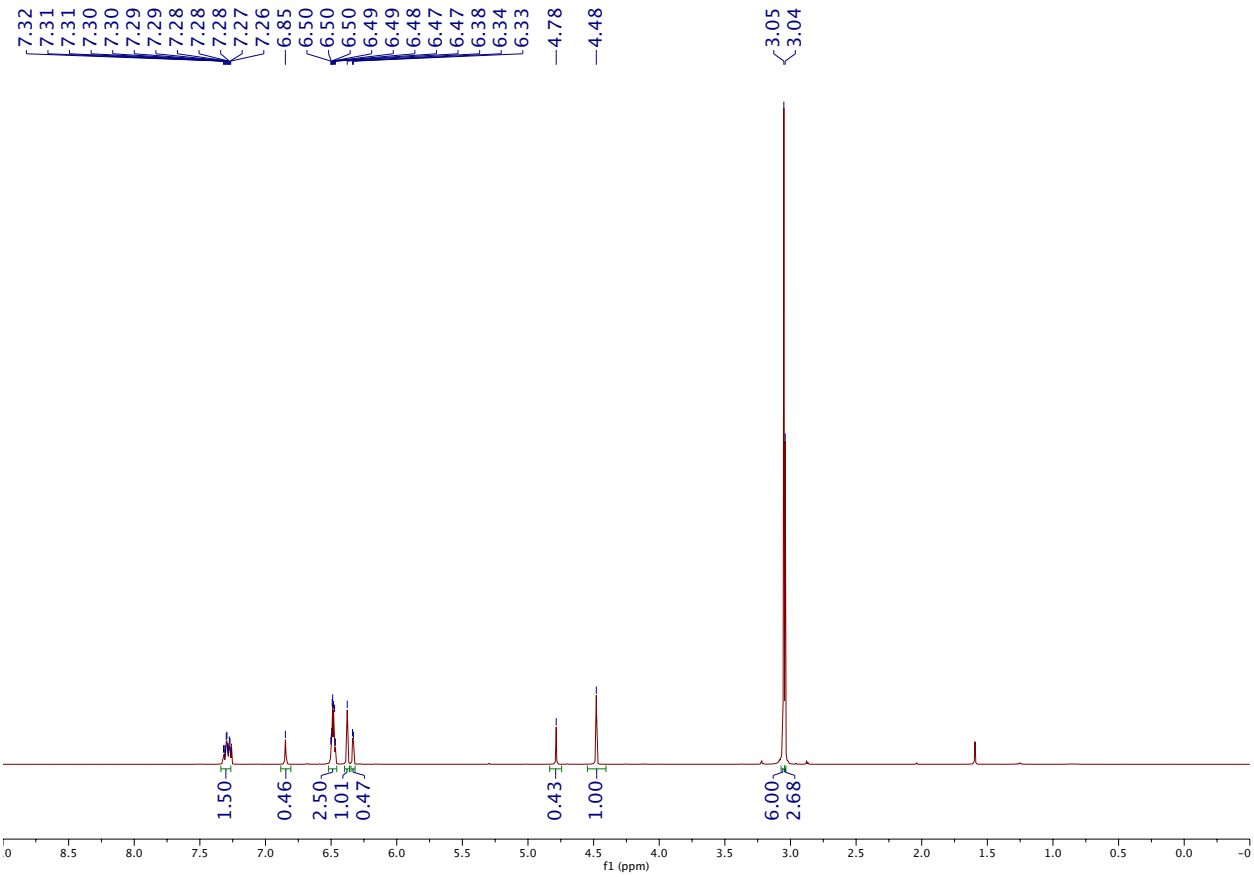
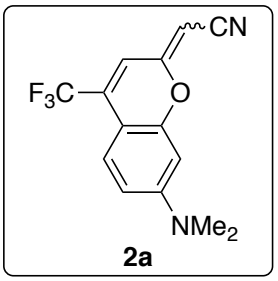
NMR Spectra



2a

^{1}H NMR

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O
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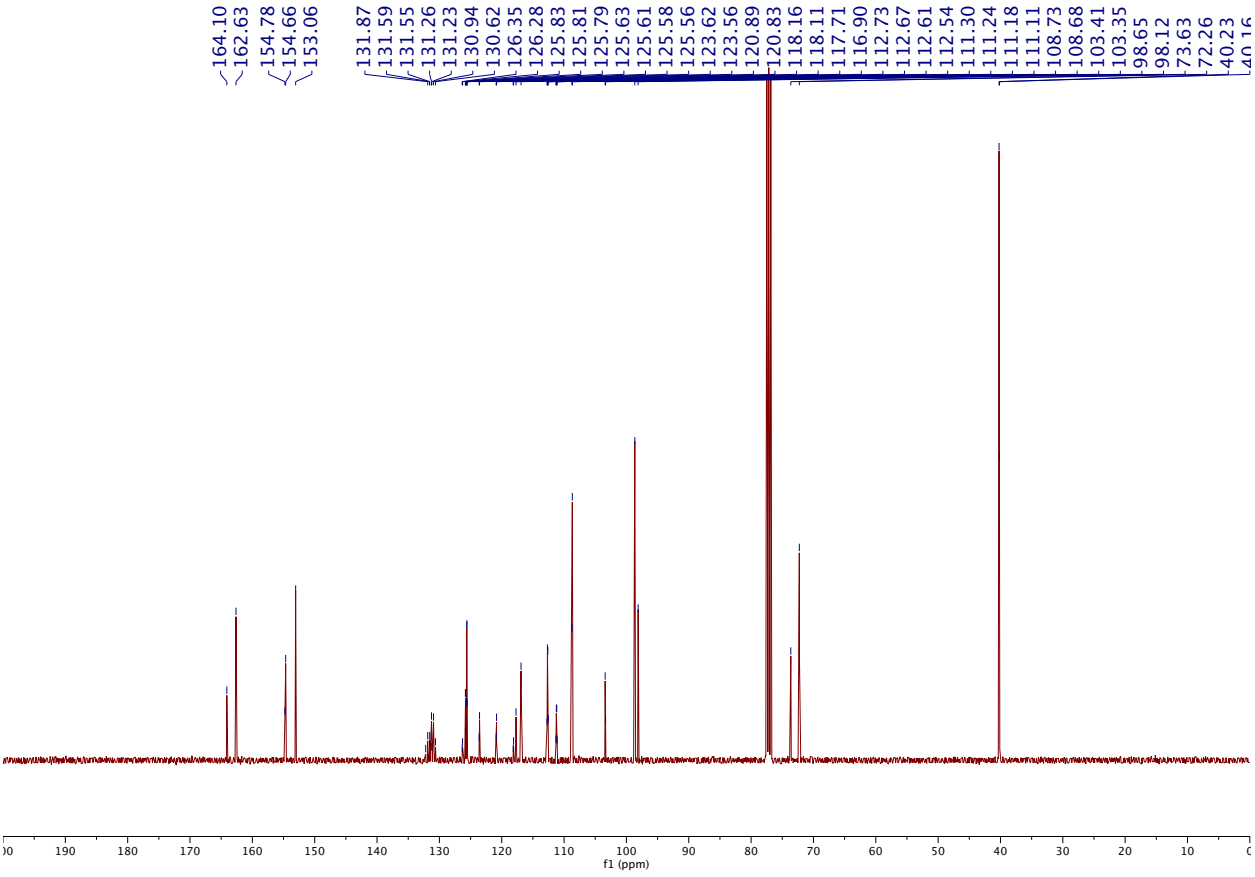
2a

^{13}C NMR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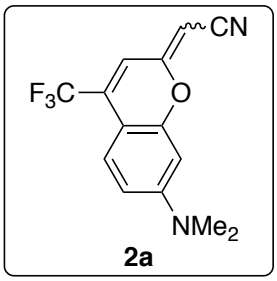
f1 (ppm)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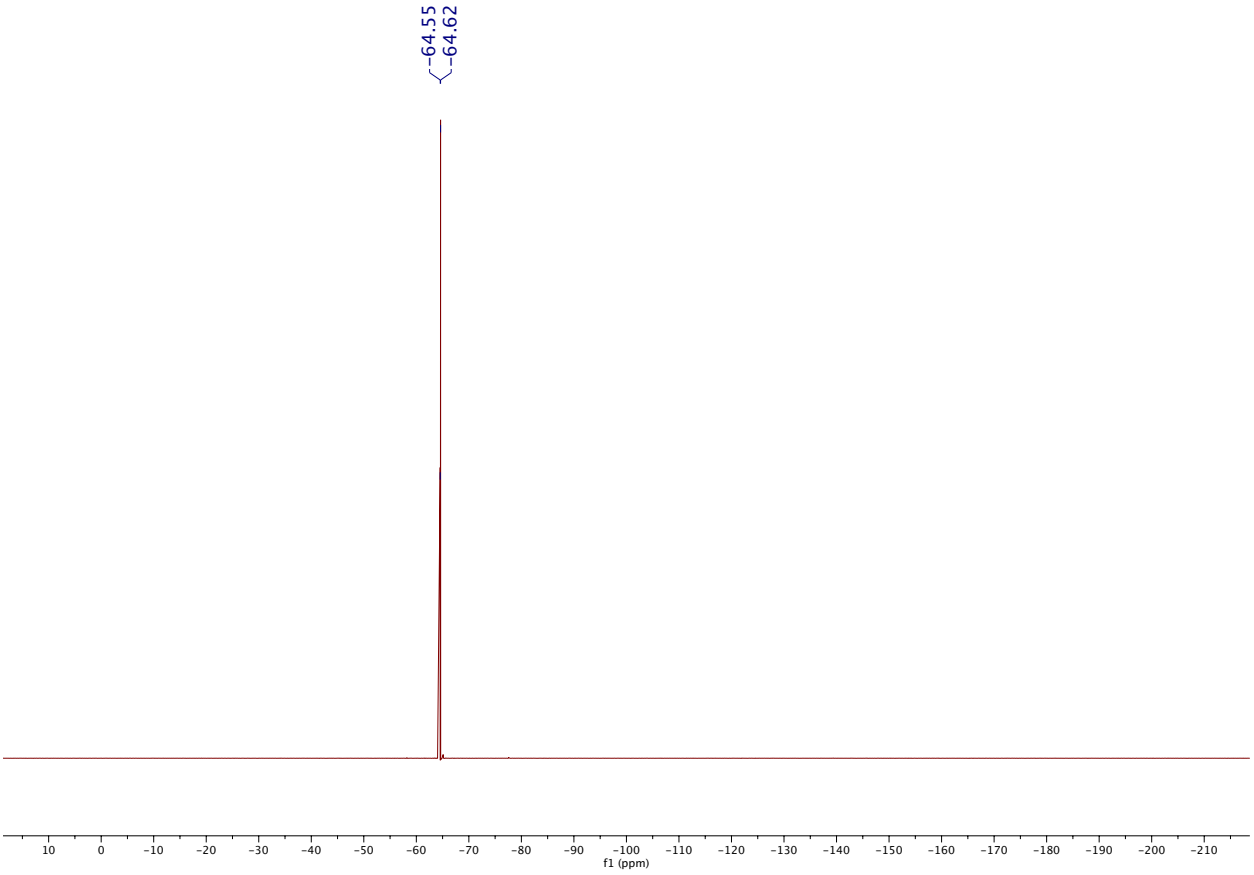
2a

^{19}F NMR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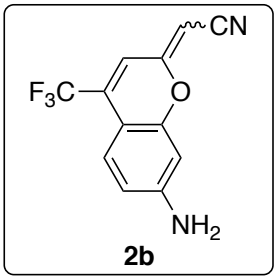
CN
F3C
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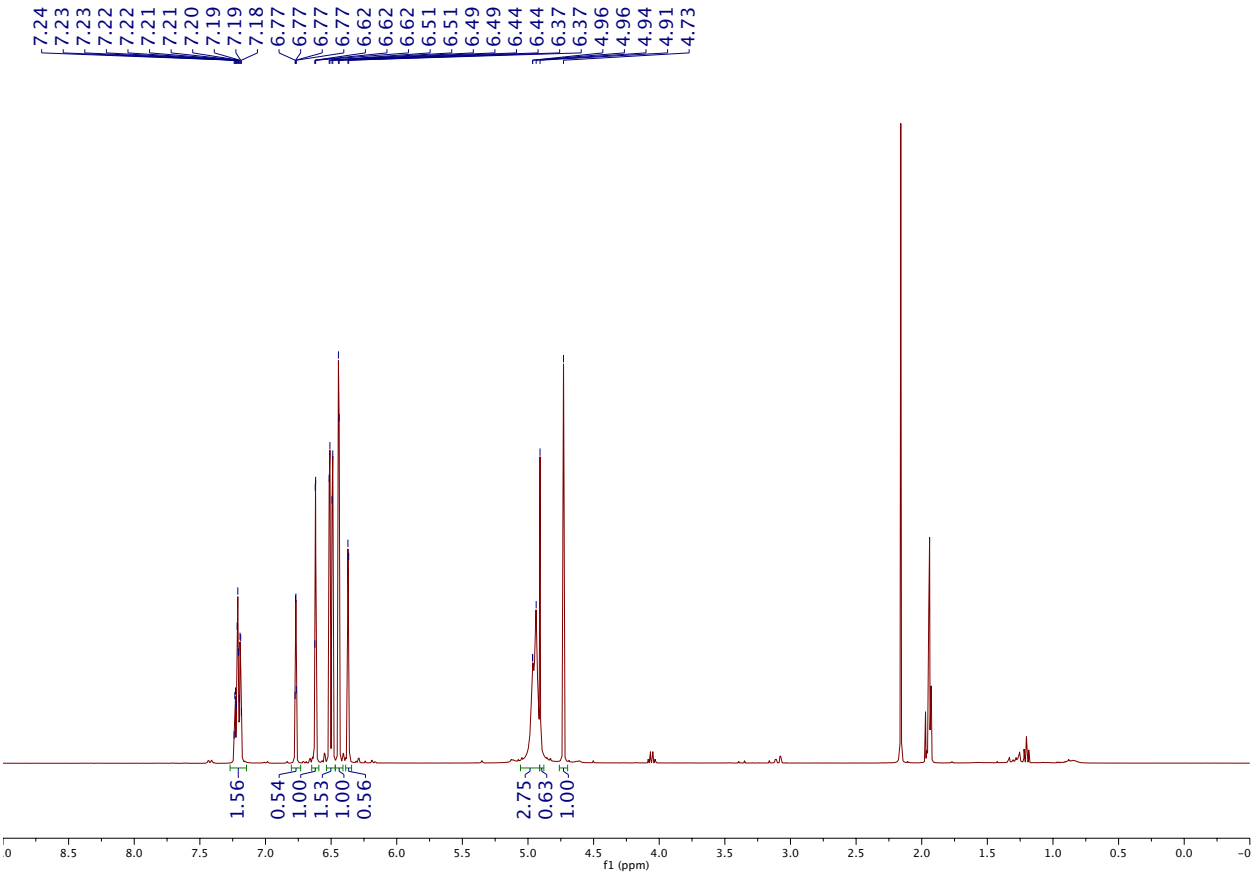
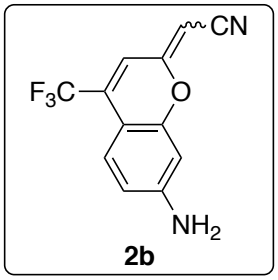
NH2

2b

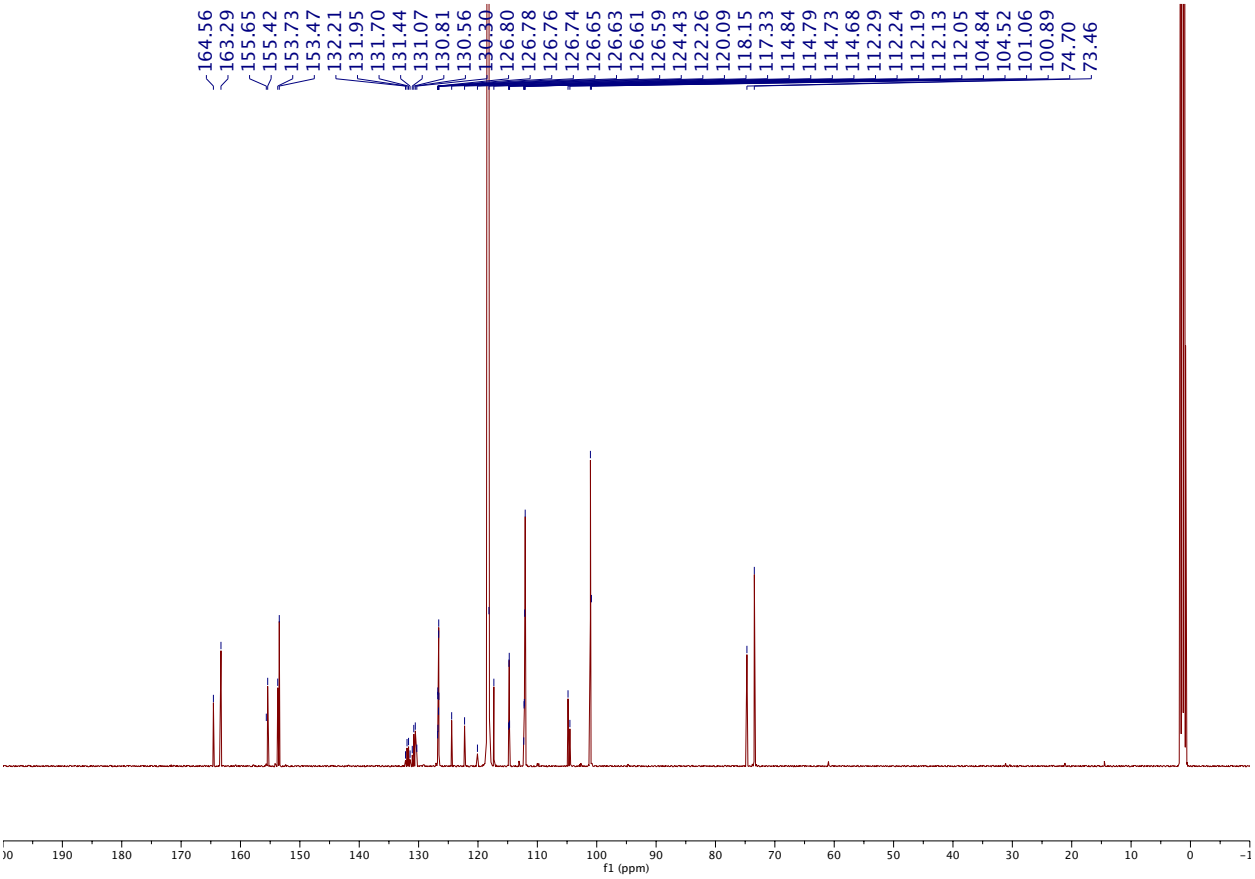
^{1}H NMR

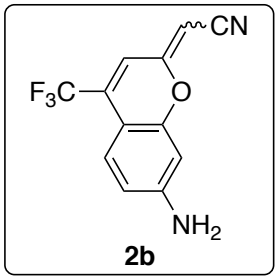

7.24
7.23
7.23
7.22
7.22
7.21
7.21
7.20
7.19
7.19
7.18
6.77
6.77
6.77
6.77
6.62
6.62
6.62
6.51
6.51
6.49
6.49
6.44
6.44
6.37
6.37
4.96
4.94
4.91
4.73
1.56
0.54
1.00
1.53
1.00
0.56
2.75
0.63
1.00
f1 (ppm)


CN
F3C
O
NH2

2b

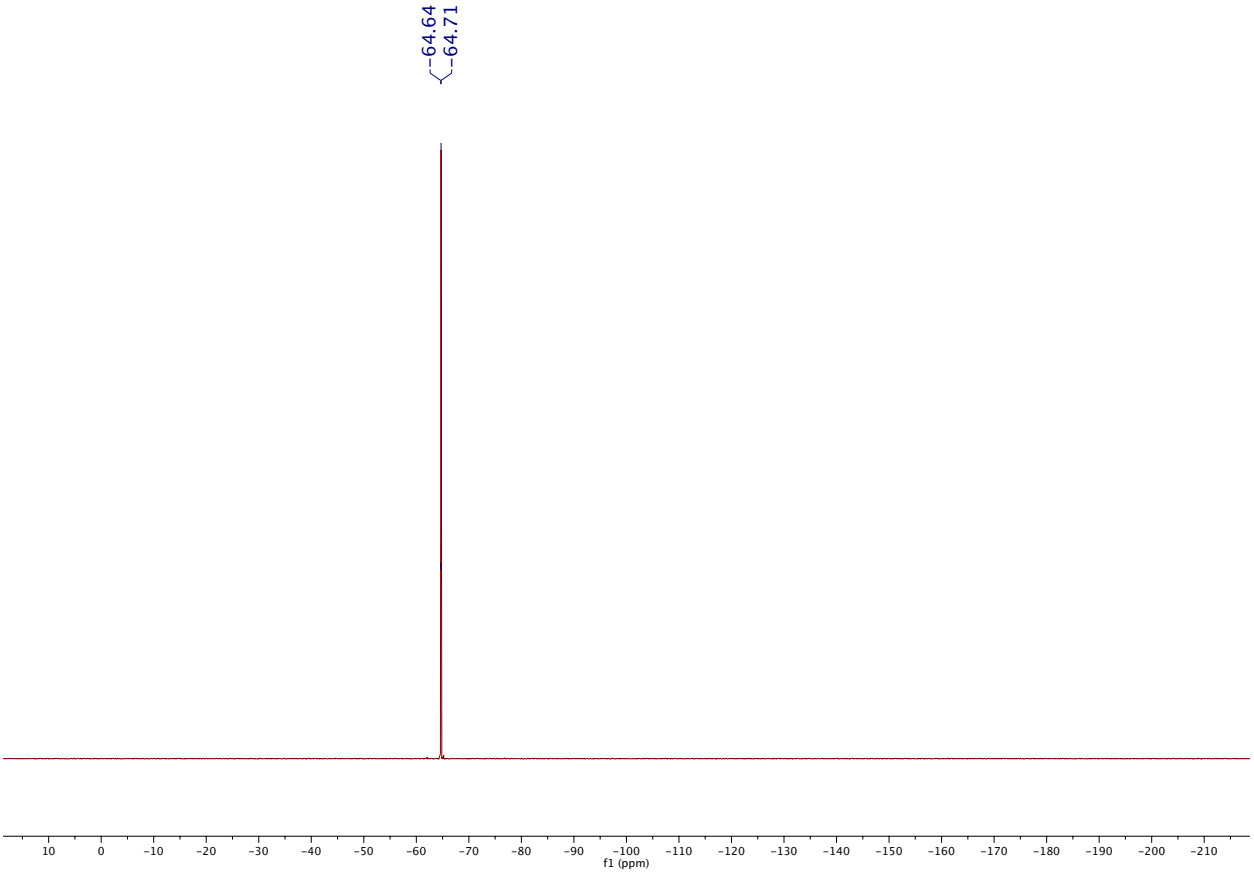
^{13}C NMR

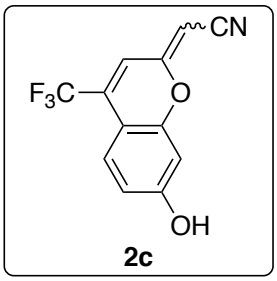

164.56
163.29
155.65
155.42
153.73
153.47
132.21
131.95
131.70
131.44
131.07
130.81
130.56
130.30
126.80
126.78
126.76
126.74
126.65
126.63
126.61
126.59
124.43
122.26
120.09
118.15
117.33
114.84
114.79
114.73
114.68
112.29
112.24
112.19
112.13
112.05
104.84
104.52
101.06
100.89
74.70
73.46
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


CN
F3C
O
NH2

2b

^{19}F NMR

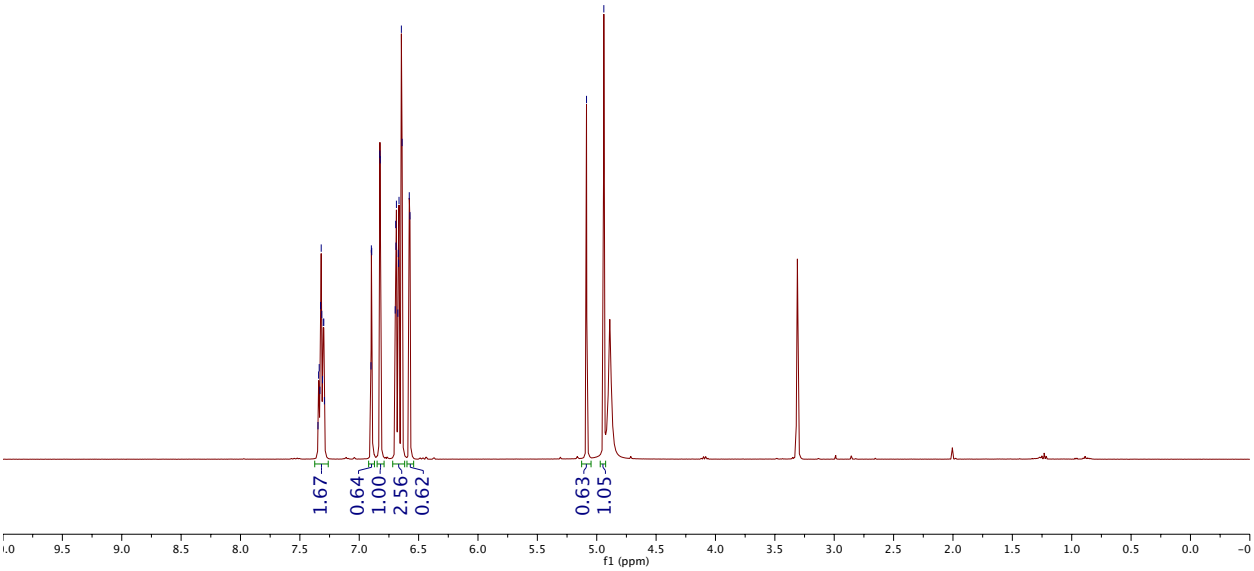

-64.64
-64.71
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-200
-210
f1 (ppm)

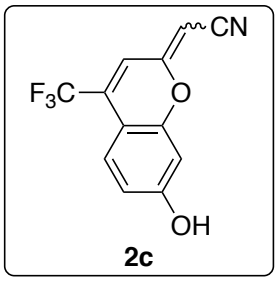

CN
F3C
O
OH

2c

^{1}H NMR

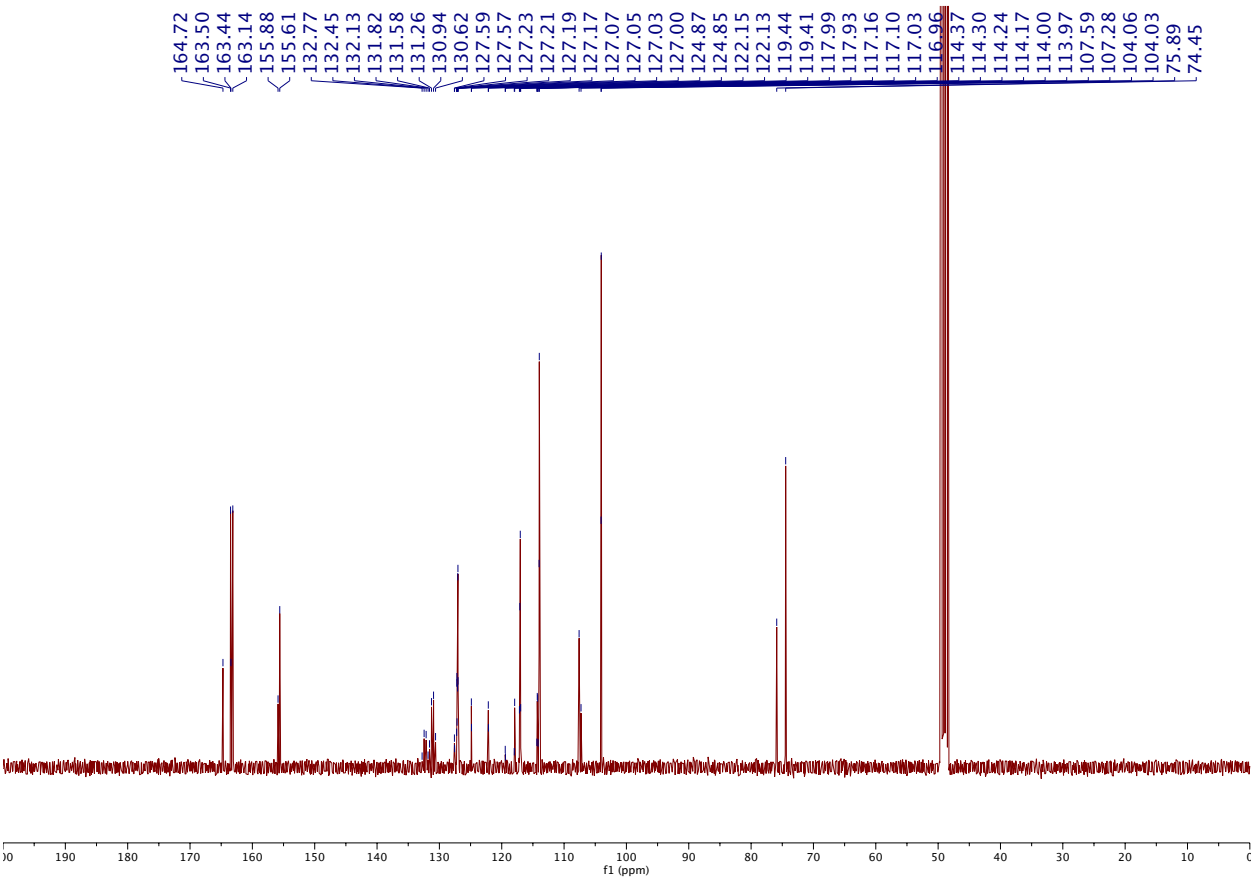

7.35
7.34
7.34
7.33
7.33
7.32
7.32
7.31
7.30
7.30
7.29
6.90
6.90
6.89
6.83
6.82
6.70
6.69
6.69
6.69
6.67
6.67
6.67
6.66
6.65
6.64
6.58
6.57
5.09
4.94

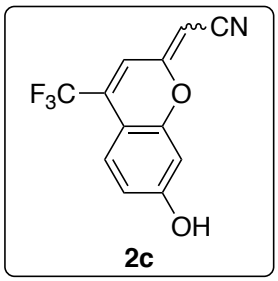

1.67
0.64
1.00
2.56
0.62
0.63
1.05
f1 (ppm)


CN
F3C
O
OH

2c

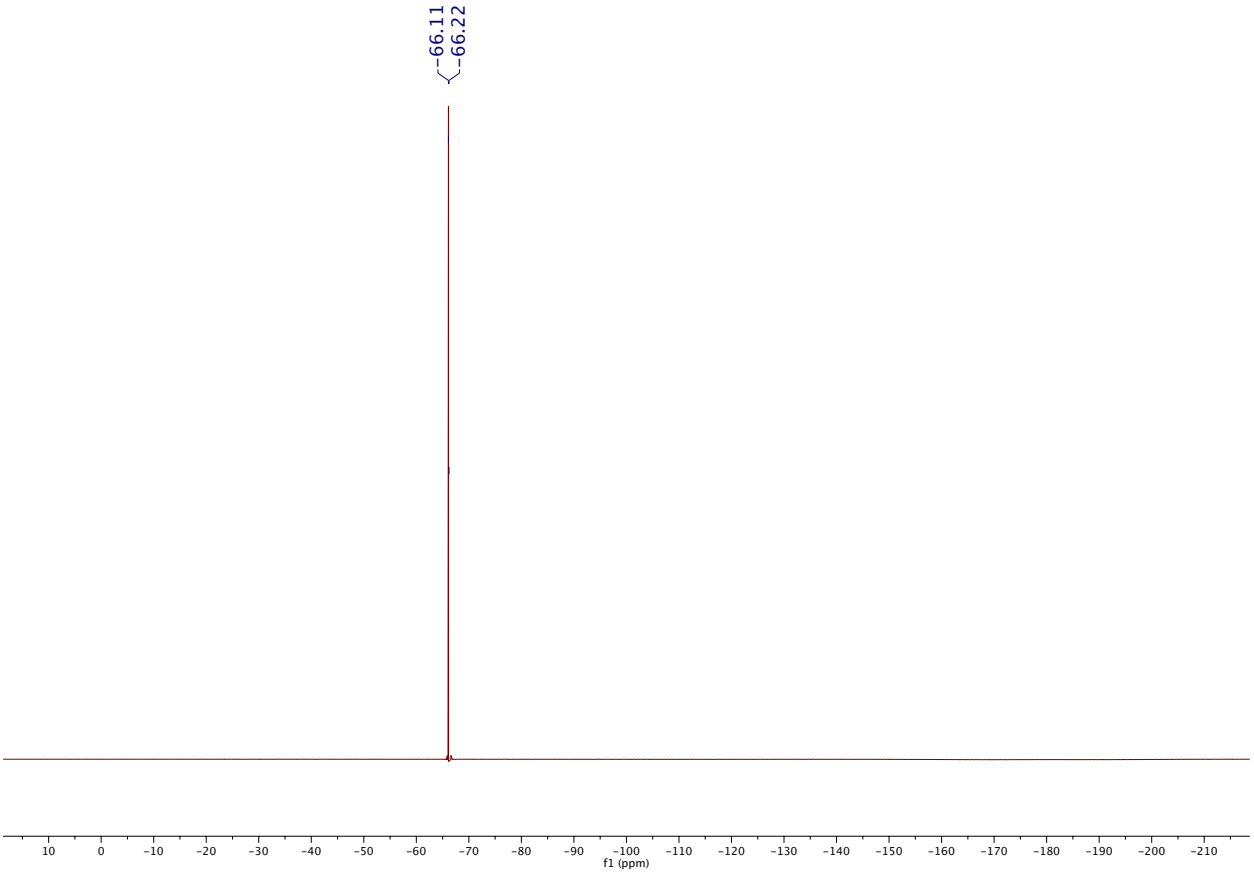
^{13}C NMR

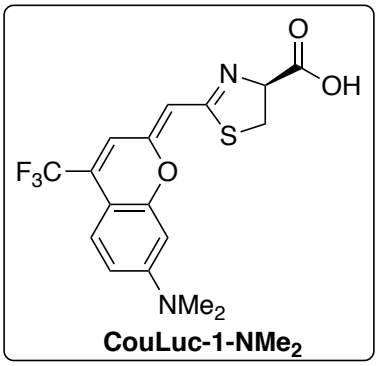

164.72
163.50
163.44
163.14
155.88
155.61
132.77
132.45
132.13
131.82
131.58
131.26
130.94
130.62
127.59
127.57
127.23
127.21
127.19
127.17
127.07
127.05
127.03
127.00
124.87
124.85
122.15
122.13
119.44
119.41
117.99
117.93
117.16
117.10
117.03
116.96
114.37
114.30
114.24
114.17
114.00
113.97
107.59
107.28
104.06
104.03
75.89
74.45
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


CN
F3C
O
OH

2c

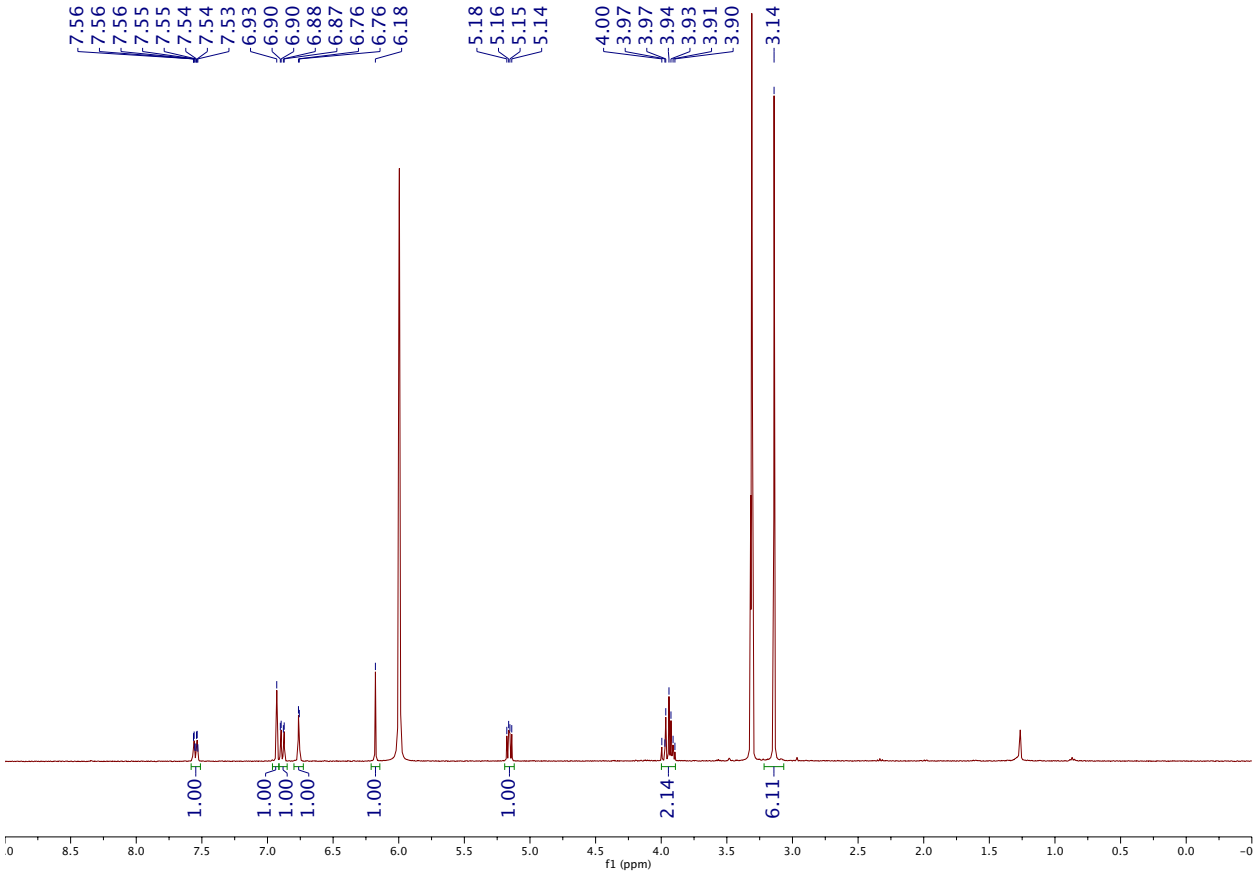
^{19}F NMR

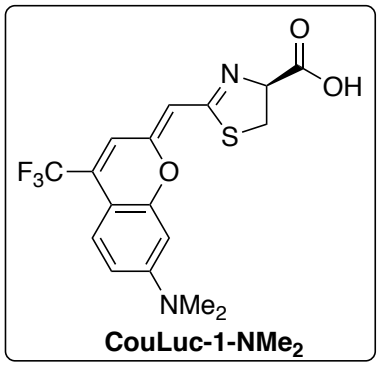

-66.11
-66.22
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-200
-210
f1 (ppm)



CouLuc-1-NMe$_2$

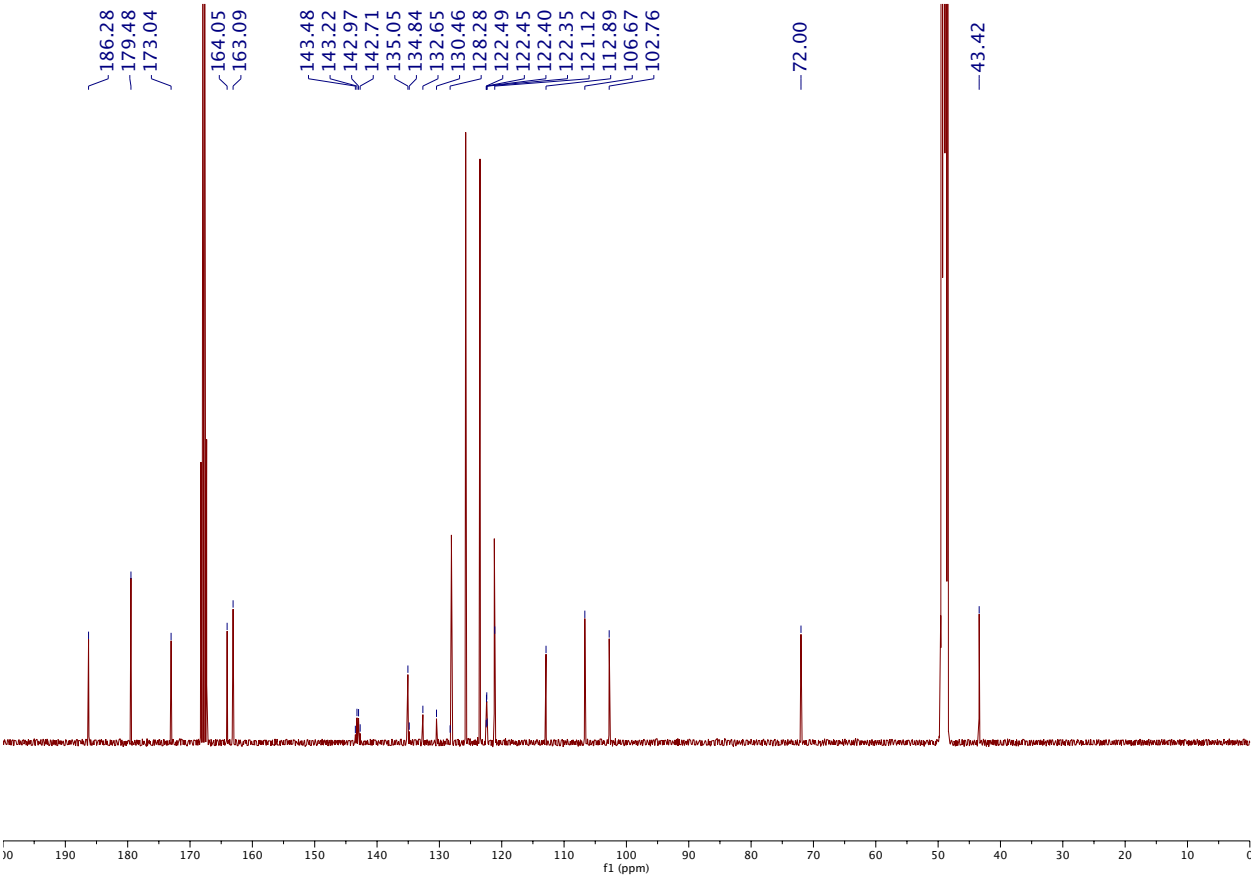
^{1}H NMR

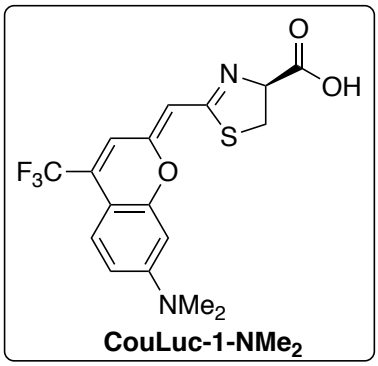




CouLuc-1-NMe$_2$

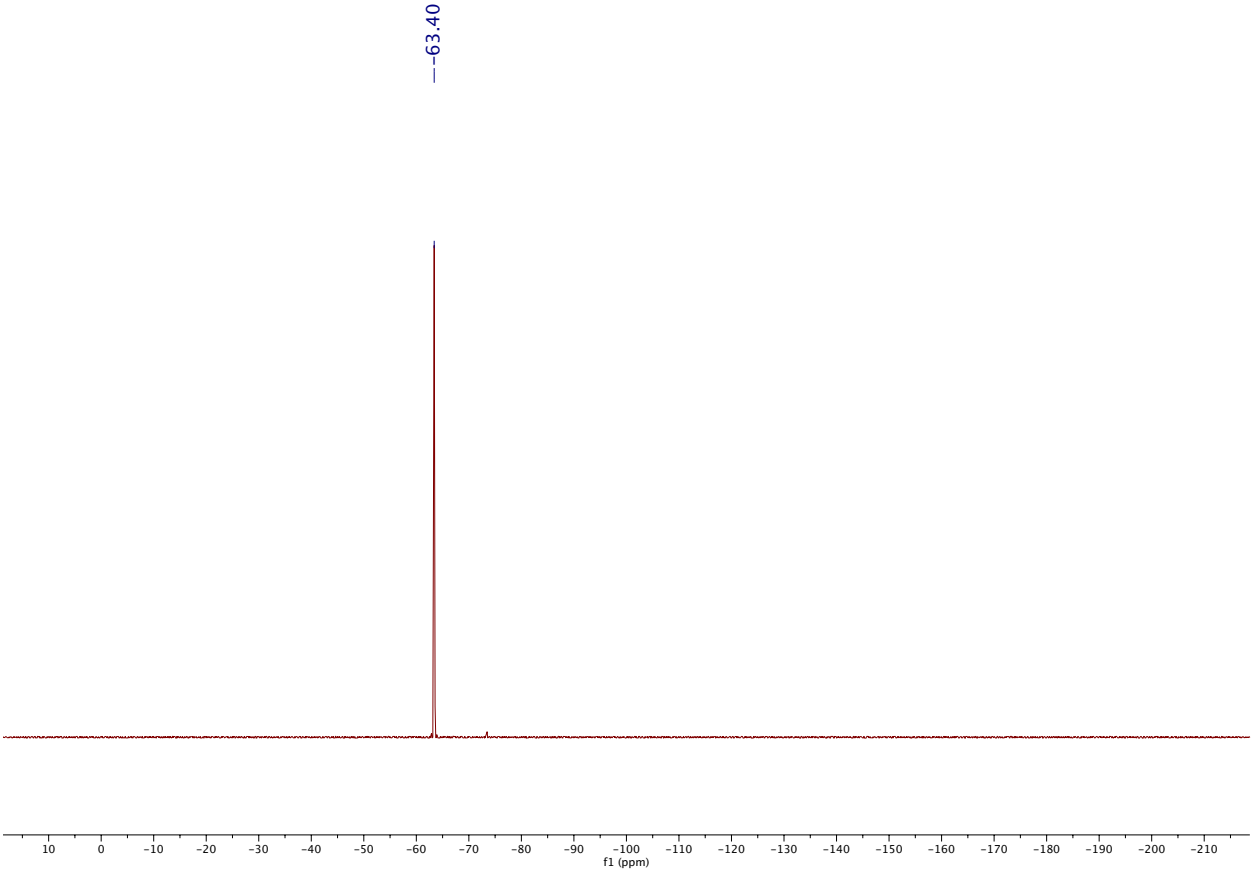
^{13}C NMR

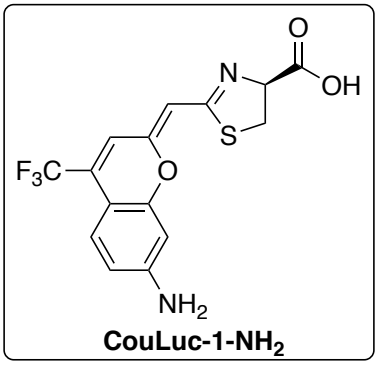




CouLuc-1-NMe$_2$

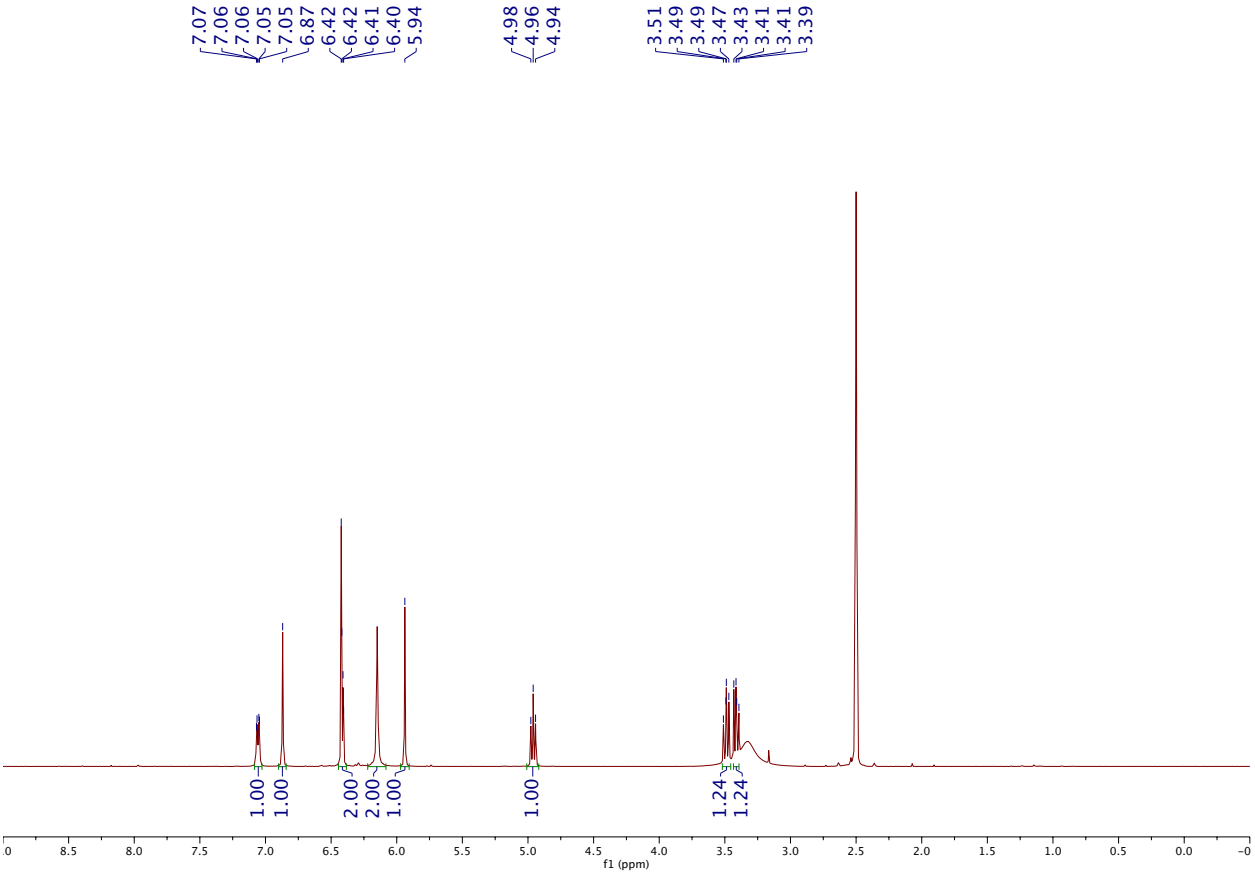
^{19}F NMR

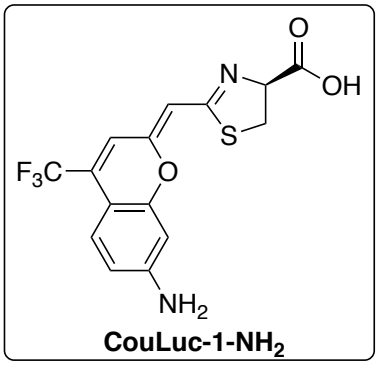




CouLuc-1-NH$_2$

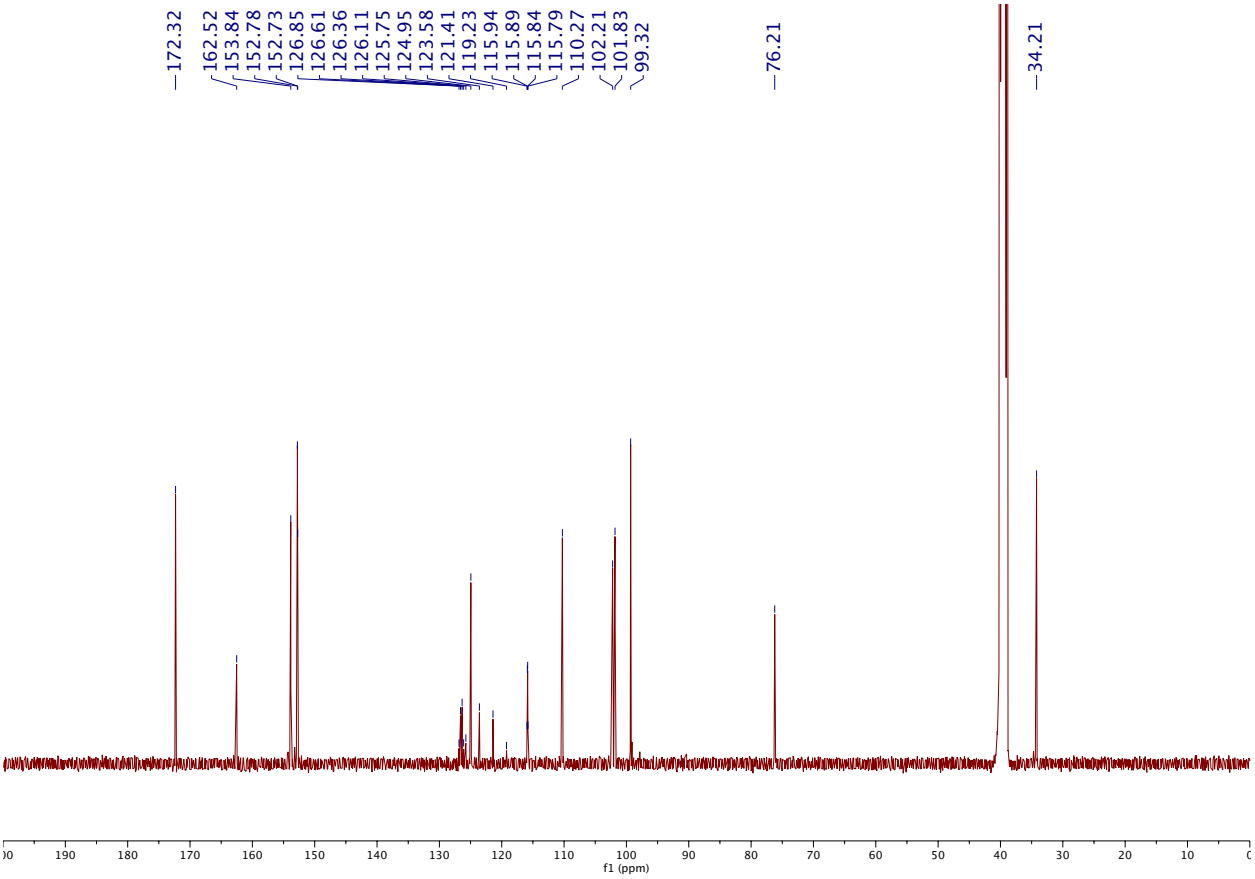
^{1}H NMR

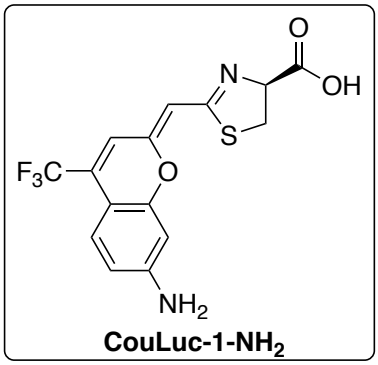




CouLuc-1-NH_2

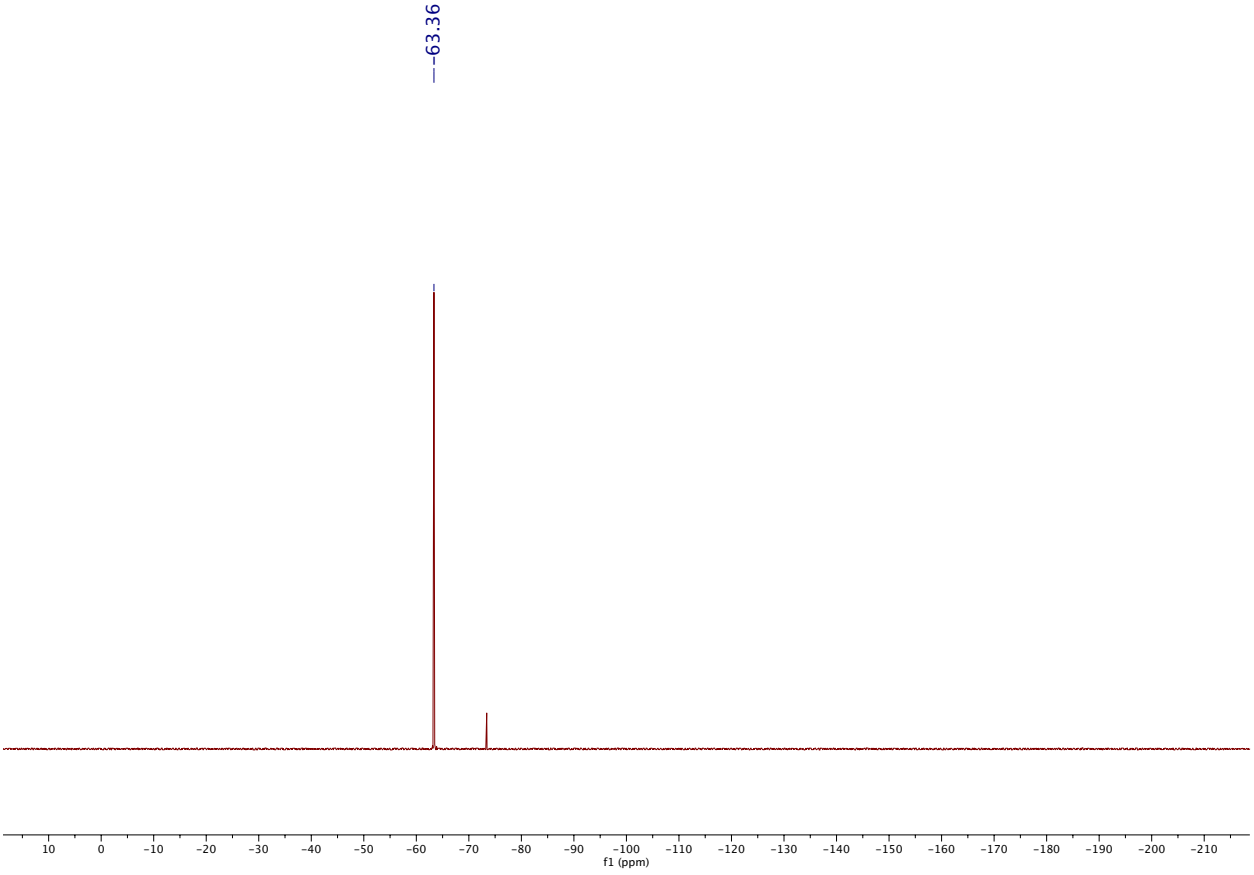
^{13}C NMR

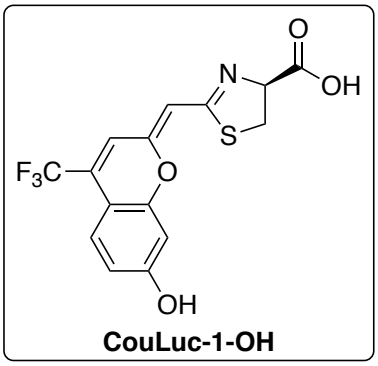




CouLuc-1-NH_2

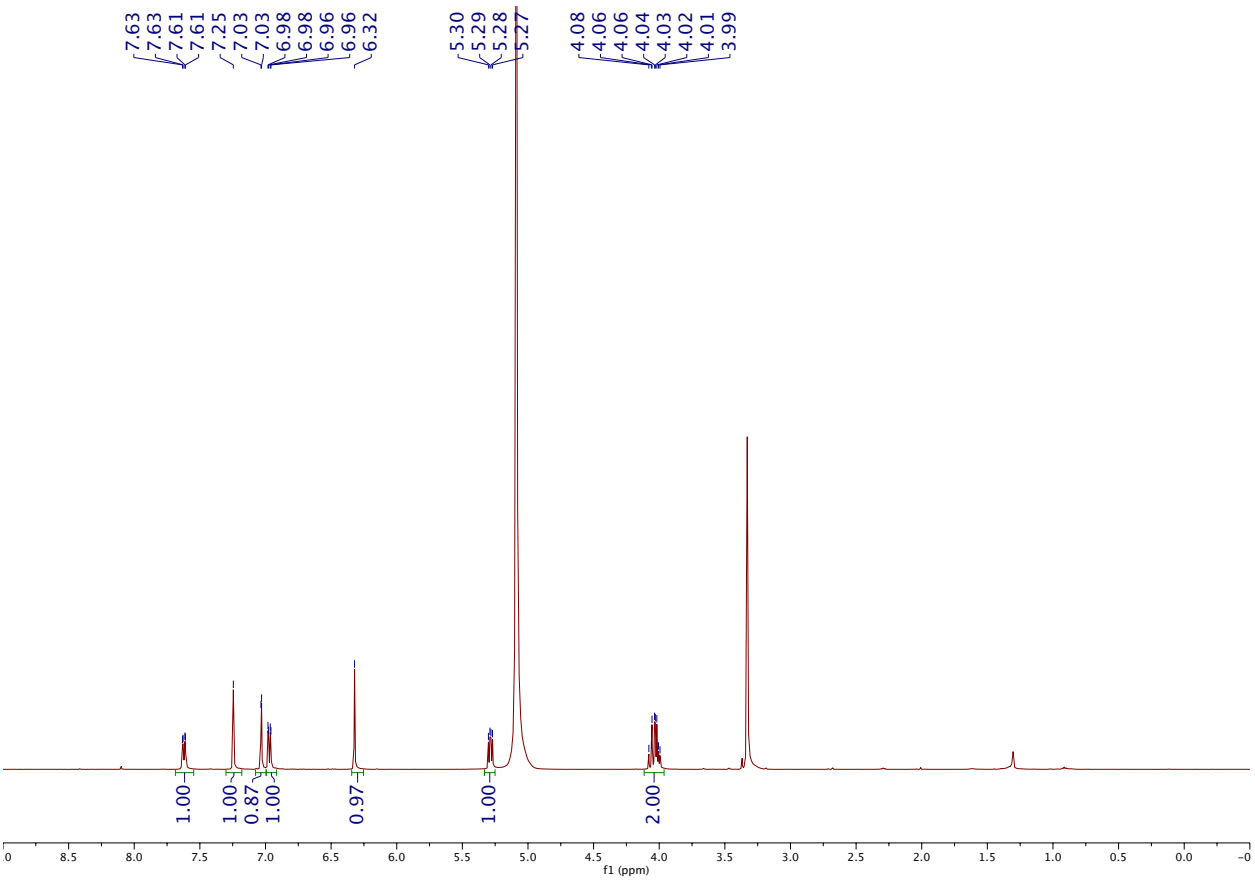
^{19}F NMR

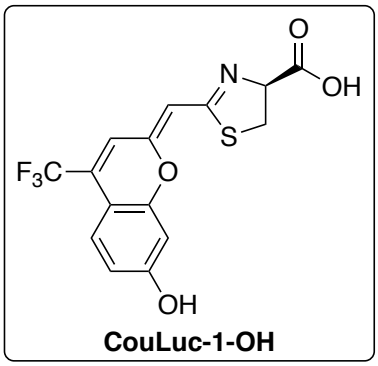




CouLuc-1-OH

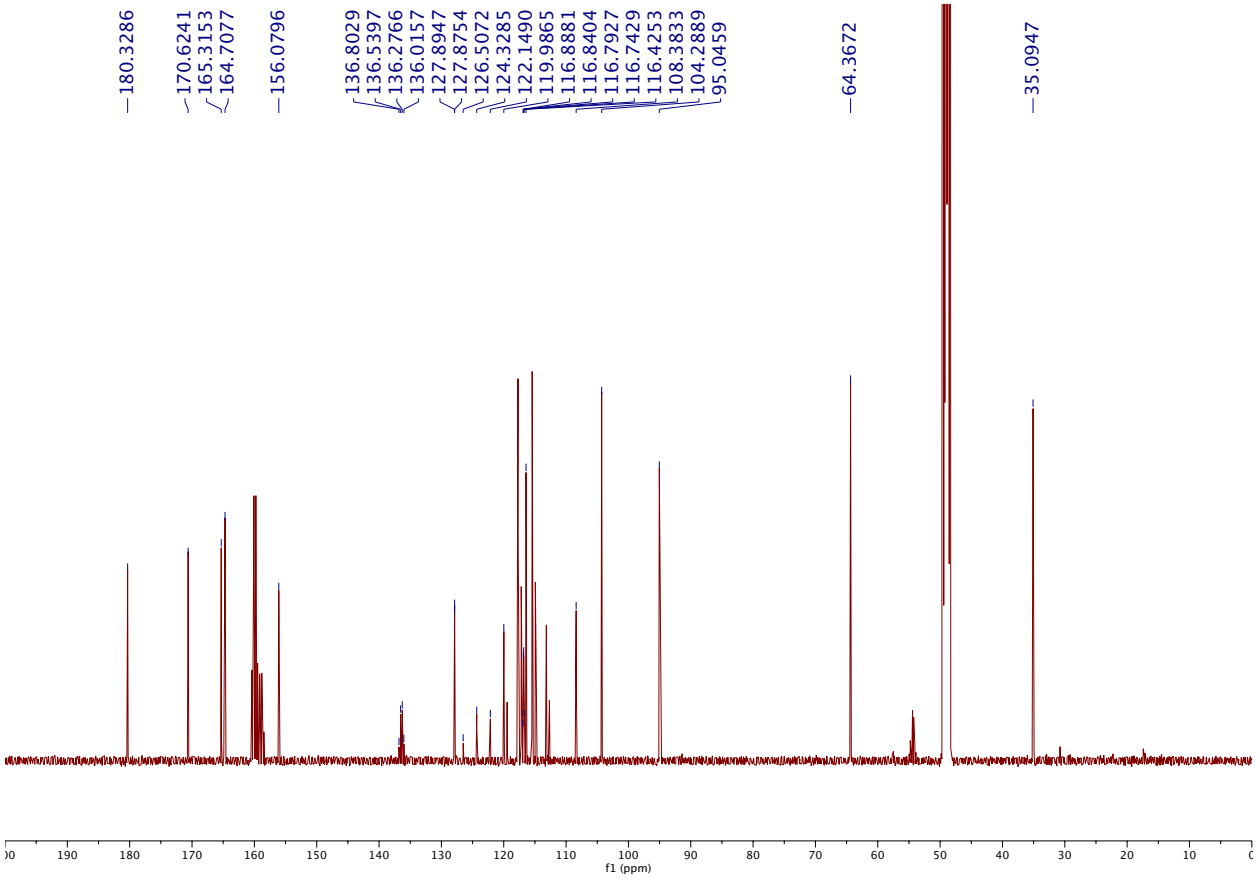
^{1}H NMR

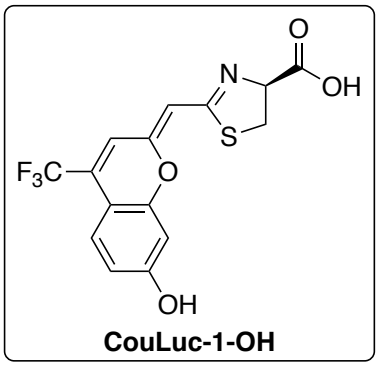




CouLuc-1-OH

^{13}C NMR





CouLuc-1-OH

^{19}F NMR

CD_3OD + TFA-d_1