Contents

I. Supplemental figures

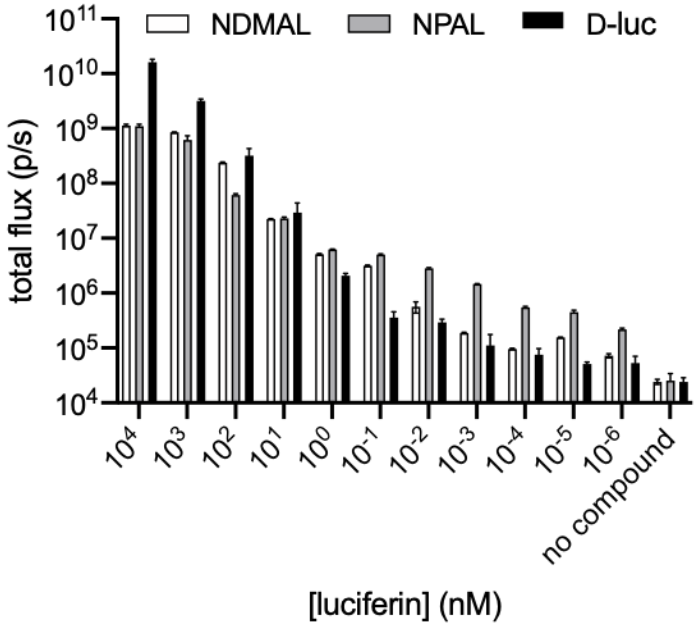


Figure S1: Light emission observed with varying concentrations of luciferin. Luciferins **NDMAL**, **NPAL**, or **D-luc** (0–10,000 nM) were incubated with purified firefly luciferase (Fluc, 1.2 µg) and ATP (1 mM) and light emission was measured. Error bars represent the standard error of the mean for n=3 measurements.

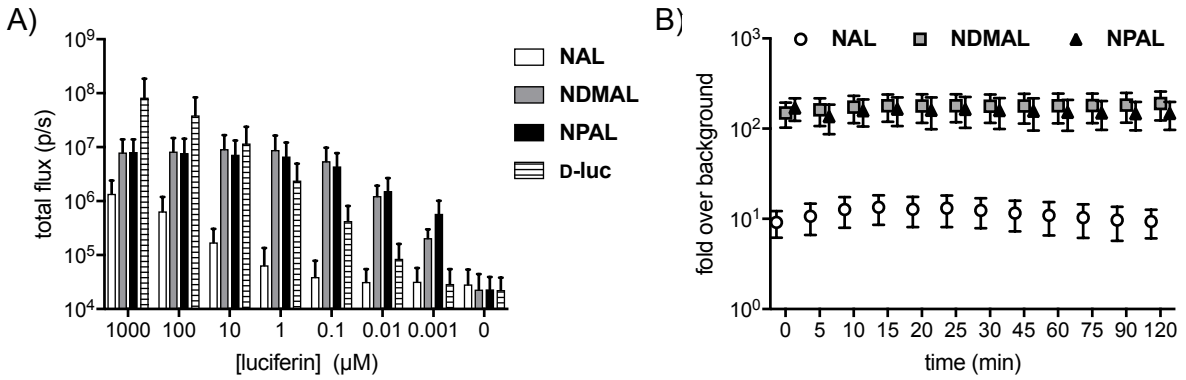


Figure S2: Cellular bioluminescence with naphthylamino luciferins in 4T1 cells. Cells (1.0 x 10^5 cells/well) stably expressing Fluc were incubated with **NAL**, **NDMAL**, **NPAL**, or **D-luc** (0–1000 µM) and imaged. A) Light emission measured after 20 min. Error bars represent the standard deviation of the mean for n=3 measurements. B) Light emission was sustained for >2 h at all concentrations examined. Data shown were measured at 100 µM. Error values represent the standard deviation of the mean for n=3 measurements.

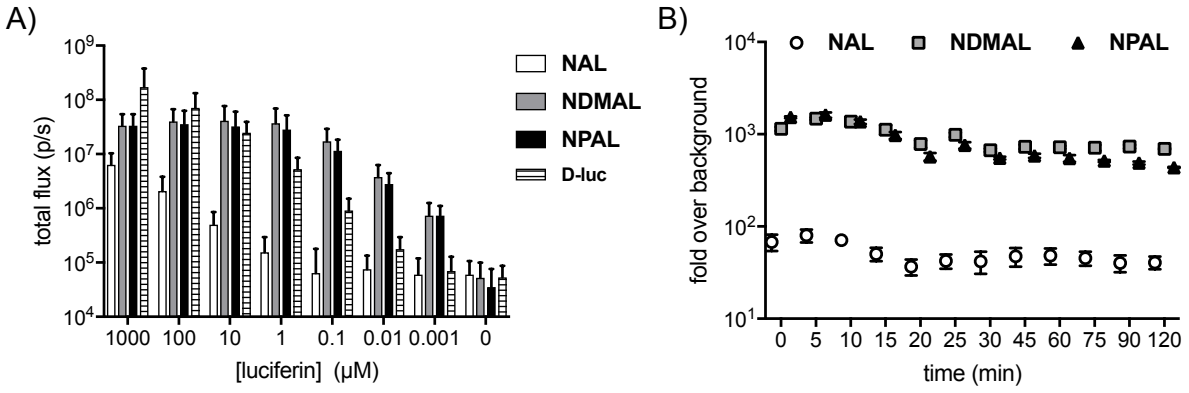


Figure S3: Light emission of naphthylamino luciferins in DB7 cells. Cells (1.0×10^5 cells/well) stably expressing Fluc were incubated with **NAL**, **NDMAL**, **NPAL**, or **D-luc** (0–1000 μM) and imaged. A) Light emission measured after 20 min. Error bars represent the standard deviation of the mean for n=3 measurements. B) Light emission was sustained for >2 h at all concentrations examined. Data shown were acquired at 100 μM. Error values represent the standard deviation of the mean for n=3 measurements.

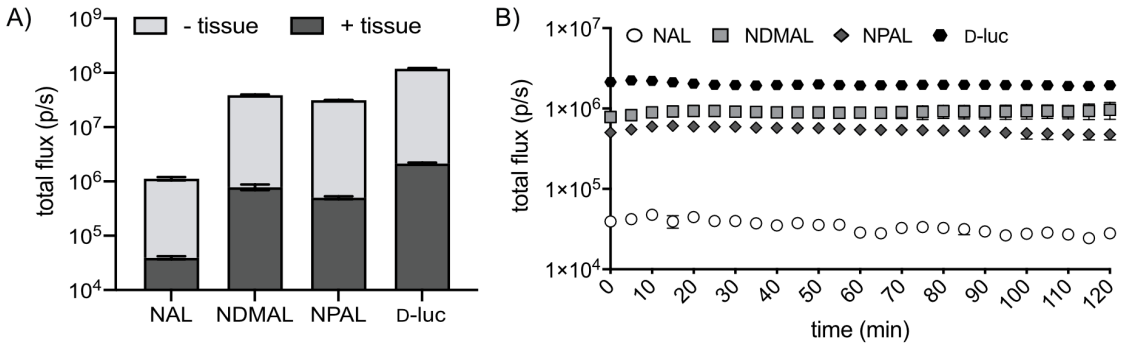
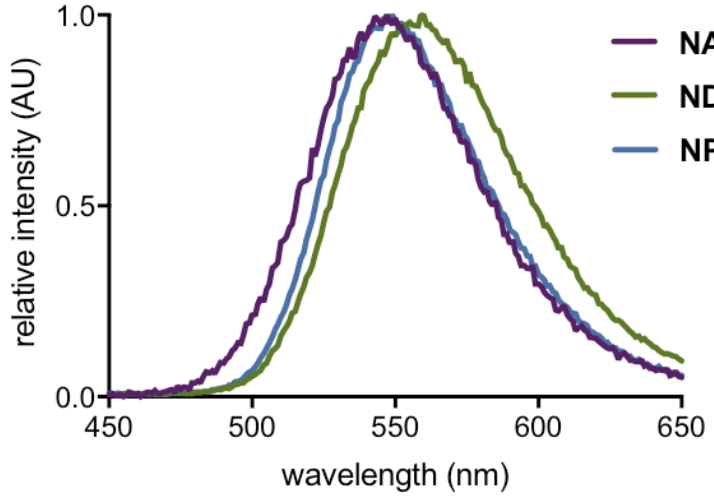


Figure S4: Light emission of naphthylamino luciferins in tissue model. DB7 cells (1.0×10^5 cells/well) stably expressing Fluc were incubated with **NAL**, **NDMAL**, **NPAL**, or **D-luc** (100 μM). A) Light emission measured in the absence or presence of tissue. Error bars represent the standard deviation of the mean for n=3 measurements. B) Light emission was sustained for >2 h. Error values represent the standard deviation of the mean for n=3 measurements.

A)



B)

analog	λ_{em} (nm)
NAL	546.5 ± 2.4
NDMAL	558.5 ± 2.5
NPAL	549.0 ± 2.2

Figure S4: Bioluminescence emission spectra. A) Each luciferin analog was incubated with Fluc in imaging buffer. Emission spectra were acquired over a range of wavelengths. B) Table of maximum emission wavelength of each luciferin analog.

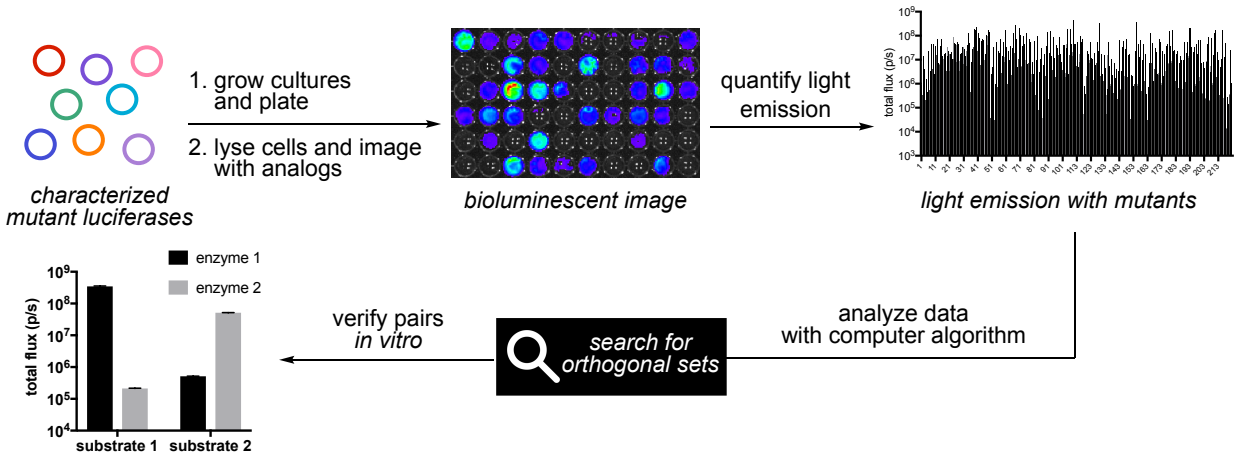
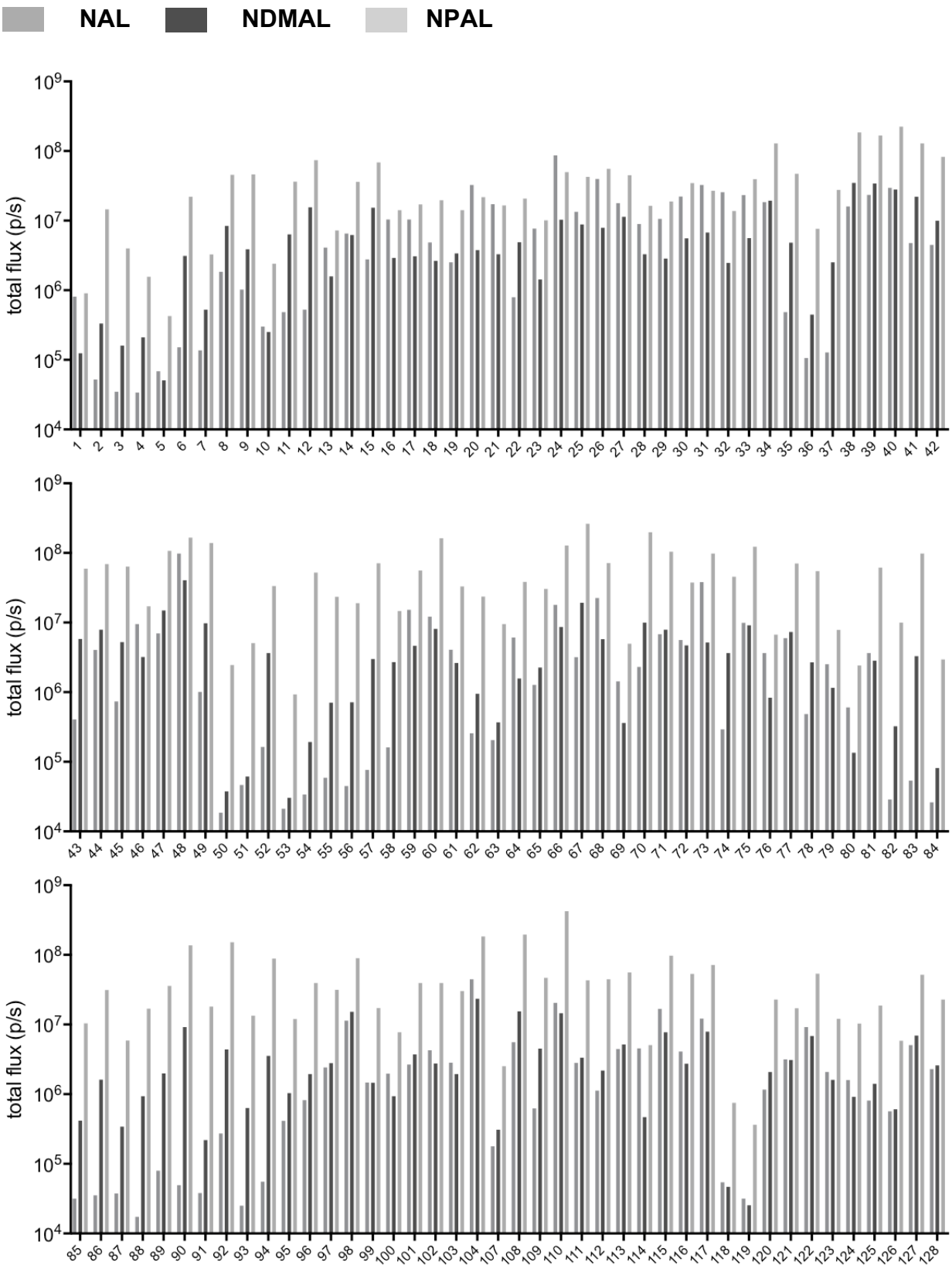


Figure S6: Screening strategy to identify luciferase mutants. Bacteria expressing mutant luciferases were expanded in 96-well plates and imaged with various luciferins. The data were compiled and analyzed using a computer algorithm to identify orthogonal pairs. The substrate selectivities of the pairs were further examined in bacterial cells.


NAL
NDMAL
NPAL
total flux (p/s)
10^9
10^8
10^7
10^6
10^5
10^4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84
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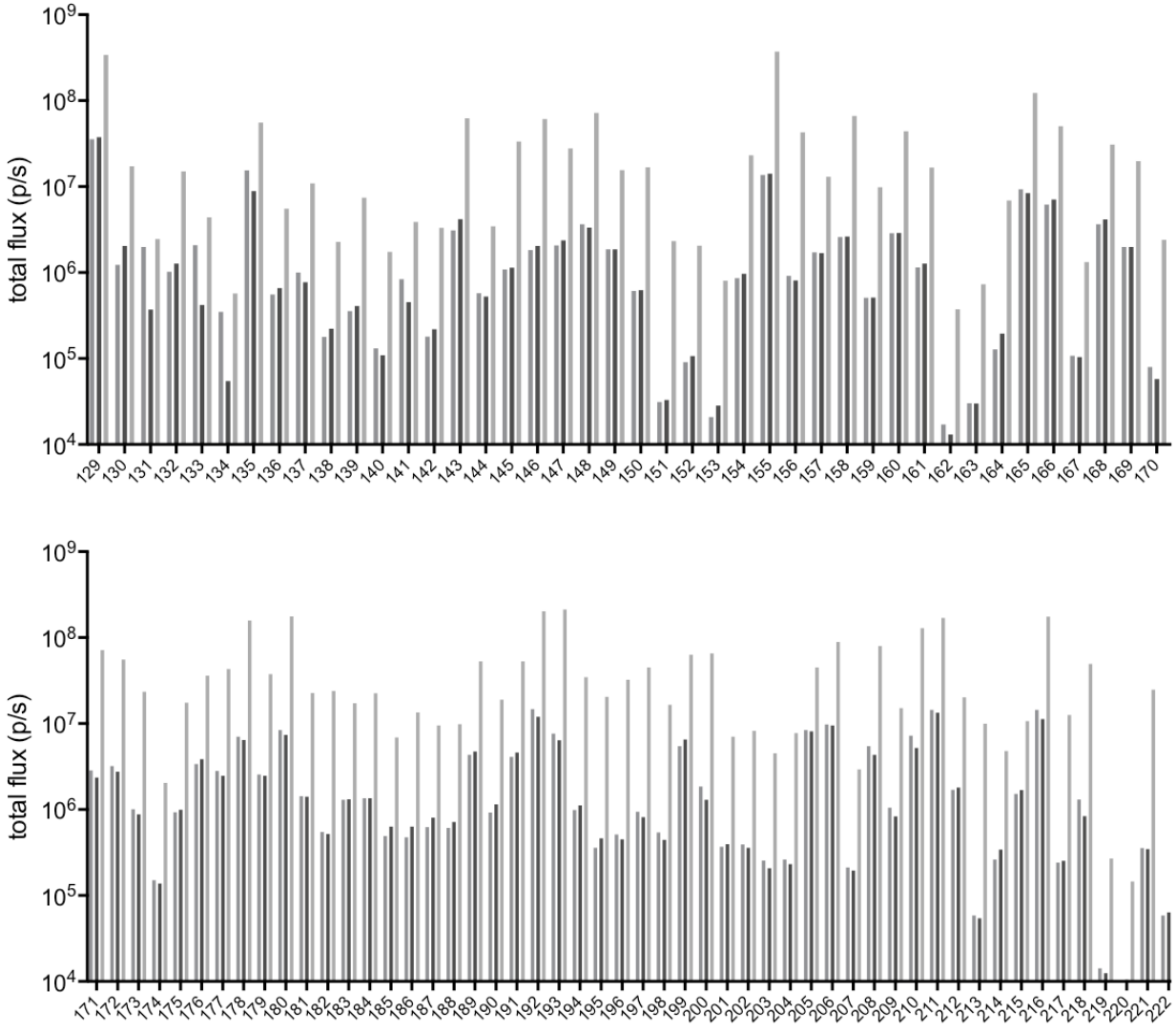


Figure S7: Screening data for naphthalene luciferins with 222 distinct mutants. Luciferase mutants were expressed in *E. coli.* The cells were lysed, distributed across 96-well plates, and imaged with 100 μM **NAL** (gray bars), **NDMAL** (dark gray bars), or **NPAL** (light gray bars).

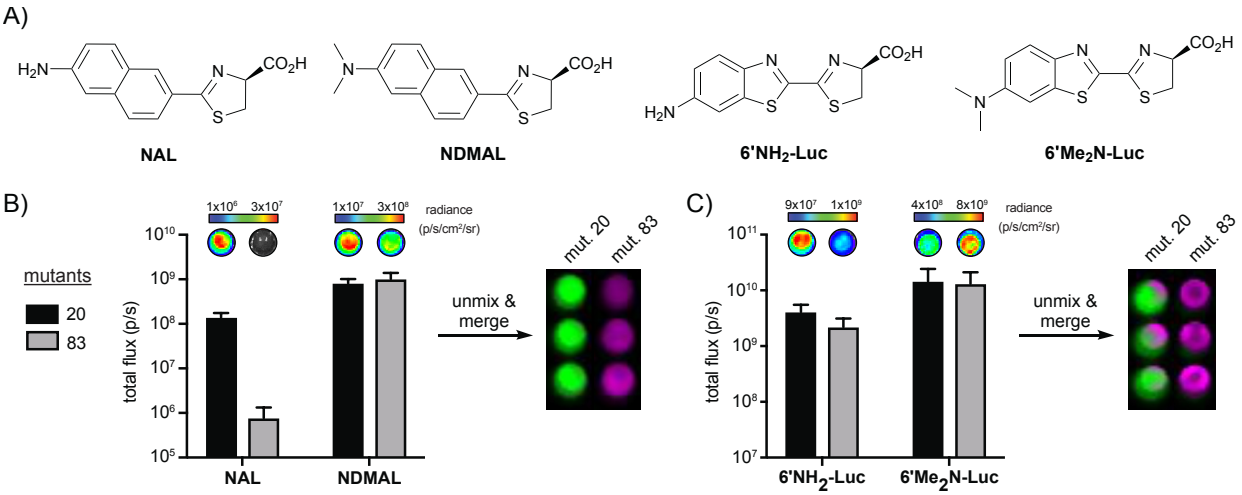
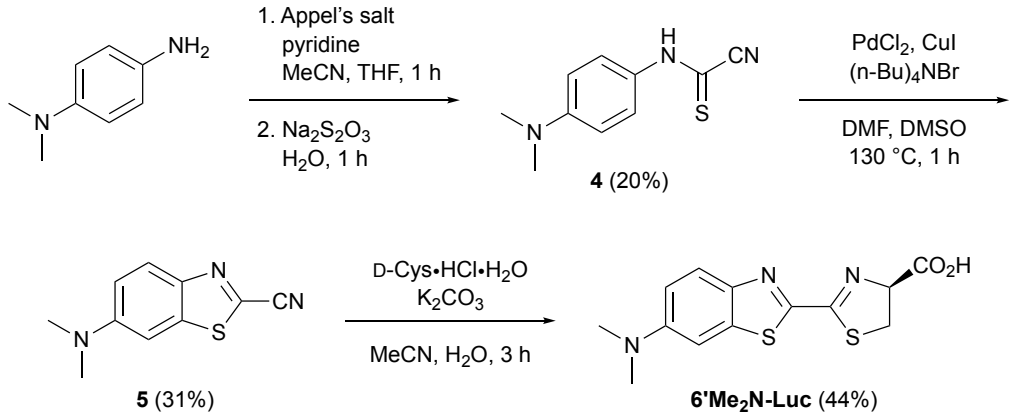


Figure S8: Naphthalene core is critical to selectivity A) Panel of luciferin analogs used in this experiment. *E. coli* cells expressing mutants 20 (black) or 83 (gray) were lysed and imaged individually with B) **NAL** and **NDMAL** or C) **6′NH$_2$-Luc** and **6′Me$_2$N-Luc**. Successful unmixing was observed only with the naphthylamino analogs. Error bars represent the standard deviation of the mean for n=3 measurements.

Table S1: Luciferase mutants used for multicomponent imaging.

mutant	mutations
20	M249L, S314T, G316T
83	R218K, S314C, G316A

Scheme S1: Synthesis of Me$_2$N-Luc



II. General biological information

General bioluminescence imaging

All assays were performed in black 96-well plates (Greiner Bio-One). Plates containing luminescent reagents were imaged in a light-proof chamber with an IVIS Lumina (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. For all assays, exposure times were set to 10–30 s, and data binning levels were set to medium. Total flux values for regions of interest were analyzed using Living Image software. Measurements were acquired in triplicate unless otherwise stated, and the data were analyzed using GraphPad Prism (version 7.0c for Macintosh, GraphPad Software).

Mammalian cell culture

HEK293, 4T1, and DB7 cells stably expressing Luc2-eGFP, Luc2, or Luc2-mRFP, respectively, were derived as described in Jones *et al.*[1] All cells were cultured in DMEM (Corning) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were maintained in a 5% CO_2 water-saturated incubator at 37 °C.

Light emission with recombinant luciferase

Recombinant Fluc was expressed and purified using methods detailed in Jones, *et al.*[1] Purified Fluc (1.2 μg), ATP (Sigma, 1 mM), reaction buffer (20 mM Tris-HCl, 0.5 mg/mL BSA, 0.1 mM

EDTA, 1 mM TCEP, 2 mM $MgSO_4$, pH 7.6), and luciferin substrates (1 nM–1 mM) were combined, in that order, to total 100 μL. Images were acquired and analyzed as described above.

Bioluminescence imaging with mammalian cells

HEK293, 4T1, or DB7 cells stably expressing Fluc (1 x 10^5 cells/well, 100 μL total volume) were plated 16 h prior to imaging. A solution of luciferin in 100 mM phosphate buffer (100 μL, 1 nM–1 mM final concentration) was added. Images were acquired and analyzed over 0–120 min as described above.

Bioluminescence imaging with mammalian cells and tissue mimic

DB7 cells stably expressing Fluc (1 x 10^5 cells/well, 100 μL total volume) were plated in 96-well plates. A solution of luciferin in 100 mM phosphate buffer (100 μL, 100 μM final concentration) was added. The plate was imaged in the presence or absence of tissue (1 mm thickness, Jennie-O turkey bacon) covering the plate. Images were acquired and analyzed over 0–120 min as described above.

Bioluminescence emission spectra

Emission spectra for **NAL**, **NDMAL**, and **NPAL** were recorded on a Cary Eclipse fluorimeter. Each luciferin (1 mM) was incubated in a cuvette (10 mm path length) with ATP (1 mM) and reaction buffer (20 mM Tris-HCl, 0.5 mg/mL BSA, 0.1 mM EDTA, 1 mM TCEP, 2 mM $MgSO_4$, pH 7.6) in a total reaction volume of 900 μL. Purified Fluc (10 μM) was added immediately prior to data acquisition. The emission slits on the instrument were adjusted to 5 nm. Emission data were

collected at 1 nm intervals from 450–650 nm at ambient temperature. Intensities were normalized to the peak emission intensity for each analog.

Luciferase mutant screening with NAL, NDMAL, NPAL

E. coli BL21 cells expressing mutant luciferases (glycerol stocks) were used to inoculate 500 µL of LB media (Genesee Scientific) supplemented with kanamycin sulfate (Kan, 40 µg/mL final concentration, Fisher Scientific). The cultures were incubated at 37 °C with shaking (250 rpm) for 16–18 h. Aliquots of the starter cultures (12 µL) were used to inoculate 400 µL of auto-induction LB media[2], and the cells were incubated at 30 °C with shaking (250 rpm) for 24 h. The cells were pelleted at 4000 rpm for 10 min, and then lysed in buffer (600 µL, 50 mM Tris-HCl, 500 mM NaCl, 0.5% (v/v) Tween® 20, 5 mM $MgCl_2$, pH 7.4). Cell lysate (90 µL) was added to black 96-well plates, followed by luciferin solution (10 µL, 100 µM luciferin, 1 mM ATP final concentration in 1% DMSO in 100 mM phosphate buffer). Bioluminescence assays were performed in duplicate. Plates were imaged and analyzed as described above.

***Orthogonal pair analysis* in vitro**

E. coli BL21 cells expressing mutant luciferases (glycerol stocks) were used to inoculate 5 mL of LB-Kan media. The cultures were incubated at 37 °C with shaking (250 rpm) for 16–18 h. Aliquots of the starter cultures (100 µL) were used to inoculate 5 mL of LB-Kan media and grown to OD_{600} = 0.8–0.9. Protein expression was induced with 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG, 2.5 µL, 500 µM final concentration, Gold Biotechnology), and the cultures were grown for 18 h with shaking (250 rpm). The cells were pelleted at 4000 rpm for 5 min, and then re-suspended in buffer (600 µL, 50 mM Tris-HCl, 500 mM NaCl, 0.5% (v/v) Tween® 20, 5 mM $MgCl_2$, pH 7.4).

Cell lysate (90 µL) was added to black 96-well plates, followed by luciferin solution (10 µL, 100 µM luciferin, 1 mM ATP final concentration in 1% DMSO in 100 mM phosphate buffer). Plates were imaged and analyzed as described above.

Sequential addition analysis with orthogonal pairs

Substrate unmixing was conducted using ImageJ (installed under the FIJI package). Luminescence images containing raw photon counts were imported into FIJI and subjected to a 2-pixel median filter. The signal at each pixel was scaled to lie between 0 and 65535 (the maximum value that can be stored in a 16-bit image). The images were stacked, and signals were unmixed using the ImageJ plugin developed by Gammon, *et al.*[3] Pseudocolors were assigned in FIJI with the "Merge Channels" tool.

III. Synthetic procedures

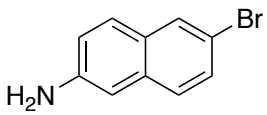
General synthetic information

All reagents and solvents were purchased from commercial suppliers and used as received. Anhydrous dimethylformamide (DMF), acetonitrile (MeCN), and tetrahydrofuran (THF) were degassed with argon and passed through two 4 x 36 in. columns of anhydrous neutral A2 (8 x 14 mesh; LaRoche Chemicals; activated at 350 °C for 12 h under flow of argon). Thin-layer chromatography (TLC) was performed using Silica Gel 60 F254-coated glass plates (0.25 mm thickness) and were visualized using UV light. Column chromatography was accomplished with Silicycle 60 Å (230-400 mesh) silica gel. Organic solutions were concentrated under reduced pressure using a Büchi rotary evaporator.

^{1}H and ^{13}C NMR spectra were obtained using either a Bruker DRX 400 (400 MHz ^{1}H, 101 MHz ^{13}C), Bruker DRX 500 equipped with a cryogenic probe (500 MHz ^{1}H, 126 MHz ^{13}C), or Bruker DRX 600 (600 MHz ^{1}H, 151 MHz ^{13}C) instrument. Spectra are internally referenced to residual solvent signals ($CDCl_3$ is referenced to 7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C, CD_3CN is referenced to 1.94 ppm for ^{1}H, $(CD_3)_2SO$ is referenced to 2.50 ppm for ^{1}H and 39.52 ppm for ^{13}C, $(CD_3)_2CO$ is referenced to 2.05 ppm for ^{1}H and 29.84 for ^{13}C). All spectra were acquired at 298 K. Chemical shifts are reported in ppm, and coupling constants (*J*) are reported in Hz. Mass spectra were acquired at the University of California, Irvine Mass Spectrometry Facility.

Compound synthesis

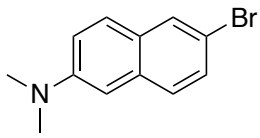
6-bromonaphthalen-2-amine (1a)



$C_{10}H_8BrN$
MW: 222.09

Following the general procedure of Takakura, *et al.,*[4] 6-bromo-2-naphthol (230. mg, 1.03 mmol) in 1,4-dioxane (5.00 mL) was added to a pressure tube. Concentrated ammonium hydroxide (NH_4OH, 5.00 mL) was added, followed by a solution of $NaHSO_3$ (2.50 g, 24.0 mmol) in H_2O (4.00 mL). The suspension was stirred at 190 °C for 16 h. The crude reaction was then cooled and concentrated *in vacuo.* The residue was extracted with ethyl acetate (EtOAc, 3 x 20 mL) and washed with brine (2 x 20 mL). The organic layers were combined and dried over magnesium sulfate ($MgSO_4$). This mixture was then filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography (eluting with 20% EtOAc in hexanes) to afford product **1a** as a white solid (180 mg, 80%). Spectral data were consistent with literature values.[4] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 1.6, 1H), 7.55 (d, *J* = 8.9, 1H), 7.48–7.39 (m, 2H), 6.97–6.89 (m, 2H), 3.86 (s, 2H).

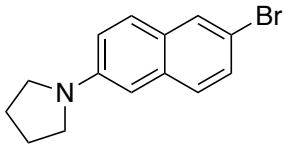
6-bromo-*N*,*N*-dimethylnaphthalen-2-amine (1b)



$C_{12}H_{12}BrN$
MW: 250.14

Following the general procedure of Takakura, *et al*.,[4] 6-bromo-2-naphthol (448 mg, 2.01 mmol) in 1,4-dioxane (10.0 mL) was added to a pressure tube. Dimethylamine (10.0 mL) of a 40 wt % solution in H_2O was added, followed by a solution of $NaHSO_3$ (4.50 g, 43.0 mmol) in H_2O (6.00 mL). The suspension was stirred at 190 °C for 16 h. The reaction was then cooled and concentrated *in vacuo*. The residue was extracted with EtOAc (3 x 30 mL) and washed with brine (2 x 30 mL). The organic layers were combined and dried over $MgSO_4$. This mixture was then filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography (eluting with 5% EtOAc in hexanes) to afford product **1b** as a white solid (280 mg, 55%). Spectral data were consistent with literature values.[4] ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (d, *J* = 2.0, 1H), 7.61 (d, *J* = 9.1, 1H), 7.52 (d, *J* = 8.7, 1H), 7.41 (dd, *J* = 8.8, 2.0, 1H), 7.17 (dd, *J* = 9.1, 2.6, 1H), 6.87 (d, *J* = 2.0, 1H), 3.05 (s, 6H).

1-(6-bromonaphthalene-2-yl)-pyrrolidine (1c)

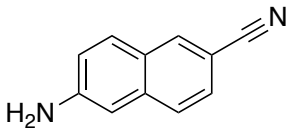


$C_{14}H_{14}BrN$
MW: 276.18

Following the general procedure of Takakura, *et al*.,[4] 6-bromo-2-naphthol (3.42 g, 15.3 mmol) in 1,4-dioxane (75.0 mL) was added to a pressure tube. Pyrrolidine (12.5 mL, 150. mmol) was added, followed by a solution of $NaHSO_3$ (15.5 g, 148 mmol) in H_2O (25.0 mL). The suspension was stirred at 190 °C for 16 h. The crude reaction was then cooled and concentrated *in vacuo*. The residue was extracted with EtOAc (3 x 100 mL) and washed with brine (2 x 100 mL). The organic layers were combined and dried over $MgSO_4$. This mixture was then filtered and

concentrated *in vacuo.* The crude product was purified by flash column chromatography (eluting with 1% EtOAc in hexanes) to afford product **1c** as a white solid (710 mg, 17%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, *J* = 2.1, 1H), 7.59 (d, *J* = 9.0, 1H), 7.49 (d, *J* = 8.8, 1H), 7.39 (dd, *J* = 8.8, 2.0, 1H), 6.99 (dd, *J* = 9.0, 2.4, 1H), 6.69 (d, *J* = 2.1, 1H), 3.40–3.37 (m, 4H), 2.08–2.03 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 146.2, 133.9, 129.6, 129.4, 128.0, 127.6, 127.3, 116.6, 114.2, 104.6, 47.9, 25.6. HRMS (ESI+) calculated for $C_{14}H_{14}BrNH$ $[M+H]^+$ = 276.0383, found 276.0387.

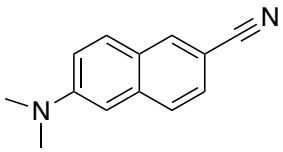
6-amino-2-naphthonitrile (2a)



$C_{11}H_8N_2$
MW: 168.20

Following the general procedure of Takakura, *et al*.,[4] compound **1a** (558 mg, 2.50 mmol) in DMF (3.00 mL) was added to a flame-dried round-bottom flask. Copper(I) cyanide (CuCN, 895 mg, 10.0 mmol) was added, and the reaction was stirred at 150 °C for 3 h. Additional CuCN was then added (455 mg, 5.00 mmol) and the reaction was stirred for 1 h at 150 °C. The reaction was then cooled, diluted with H_2O (25 mL) and poured into a separatory funnel containing 10% ethylenediamine in H_2O (25 mL). The solution was extracted with EtOAc (3 x 50 mL). The organic layers were combined and dried over $MgSO_4$. This mixture was filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography (eluting with 30% EtOAc in hexanes) to afford product **2a** as a yellow solid (85 mg, 20%). Spectral data were consistent with literature values.[4] ^{1}H NMR (400 MHz, CD_3CN) δ 8.09 (d, *J* = 1.3, 1H), 7.69 (d, *J* = 8.8, 1H), 7.62 (d, *J* = 8.9, 1H), 7.45 (dd, *J* = 8.6, 1.7, 1H), 7.06 (dd, *J* = 8.8, 2.3, 1H), 6.93 (d, *J* = 2.2, 1H), 4.78 (s, 2H).

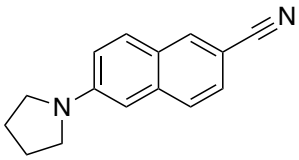
6-(dimethylamino)-2-naphthonitrile (2b)



$C_{13}H_{12}N_2$
MW: 196.25

Following the general procedure of Takakura, *et al*.,[4] compound **1b** (267 mg, 1.07 mmol) was added in DMF (1.50 mL) to a flame-dried round-bottom flask. CuCN (356 mg, 3.97 mmol) was added, and the reaction was stirred at 150 °C for 3 h. Additional CuCN was then added (100 mg, 1.10 mmol) and the reaction was stirred for 1 h at 150 °C. The reaction was then cooled, diluted with H_2O (5 mL) and poured into a separatory funnel containing 10% ethylenediamine in H_2O (20 mL). The solution was extracted with EtOAc (3 x 30 mL). The organic layers were combined and dried over $MgSO_4$. This mixture was then filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography (eluting with 5% EtOAc in hexanes) to afford product **2b** as a yellow solid (98 mg, 47%). Spectral data were consistent with literature values.[4] ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (s, 1H), 7.71 (d, J = 9.1, 1H), 7.63 (d, J = 8.6, 1H), 7.45 (dd, J = 8.6, 1.7, 1H), 7.19 (dd, J = 9.1, 2.6, 1H), 6.84 (d, J = 2.5, 1H), 3.11 (s, 6H).

6-(pyrrolidine-1-yl)-20-naphthonitrile (2c)

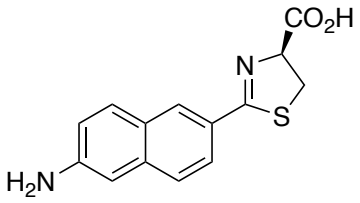


$C_{15}H_{14}N_2$
MW: 222.29

Following the general procedure of Takakura, *et al*.,[4] compound **1c** (504 mg, 1.80 mmol) in DMF (1.50 mL) was added to a flame-dried round-bottom flask. CuCN (811 mg, 9.10 mmol) was added, and the reaction was stirred at 150 °C for 3 h. Additional CuCN was then added (820 mg, 9.20 mmol) and the reaction was stirred for 1 h at 150 °C. The reaction was then cooled, diluted with H_2O (10 mL) and poured into a separatory funnel containing 10% ethylenediamine in H_2O (40 mL). Solution was extracted with EtOAc (3 x 50 mL). The organic layers were combined and dried over $MgSO_4$. This mixture was then filtered and concentrated *in vacuo.* The crude product was purified by flash chromatography (eluting with 1% EtOAc in hexanes) to afford

product **2c** as a yellow solid (140 mg, 36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (s, 1H), 7.65 (d, *J* = 9.0, 1H), 7.57 (d, *J* = 8.6, 1H), 7.40 (dd, *J* = 8.6, 1.7, 1H), 7.01 (dd, *J* = 9.0, 2.4, 1H), 6.66 (d, *J* = 2.2, 1H), 3.42–3.39 (m, 4H), 2.10–2.04 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 147.8, 137.0, 134.0, 129.6, 126.8, 126.5, 124.7, 120.6, 117.0, 104.3, 103.1, 47.7, 25.5. HRMS (ESI+) calculated for $C_{15}H_{14}N_2H$ $[M+H]^+$ = 223.1230, found 223.1231.

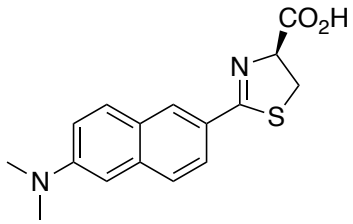
(S)-2-(6-aminonaphthalen-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (NAL)



$C_{14}H_{12}N_2O_2S$
MW: 272.32

Following the general procedure of Takakura, *et al*.,[4] compound **2a** (75.8 mg, 1.09 mmol) in ethanol (EtOH, 5.00 mL) was added to a round-bottom flask. D-Cysteine hydrochloride monohydrate (D-Cys•HCl•H_2O, 894 mg, 5.11 mmol) was dissolved in H_2O (5.00 mL). This solution was adjusted to pH 8 using solid potassium carbonate (K_2CO_3) and added to the flask containing **2a**. The resulting suspension was stirred at 80 °C for 16 h. The reaction was then cooled and concentrated *in vacuo*. The crude mixture was resuspended in H_2O (10 mL) and washed with EtOAc (2 x 10 mL) to remove unreacted starting material. The aqueous solution was acidified to pH 2 with 1 M sodium bisulfate monohydrate, and the resulting precipitate was filtered to afford **NAL** as an orange solid (140 mg, 45%). Spectral data were consistent with literature values.[4] ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 8.03 (d, *J* = 1.8, 1H), 7.77–7.72 (m, 2H), 7.54 (d, *J* = 8.7, 1H), 7.00 (dd, *J* = 8.8, 2.2, 1H), 6.85 (d, *J* = 1.8, 1H), 5.27 (dd, *J* = 9.3, 8.1, 1H), 3.72–3.58 (m, 2H).

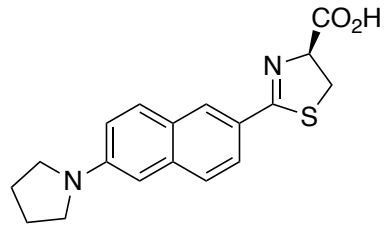
(S)-2-(6-(dimethylamino)-naphthalen-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (NDMAL)



$C_{16}H_{16}N_2O_2S$
MW: 300.38

Following the general procedure of Takakura, *et al*.,[4] compound **2b** (111 mg, 0.566 mmol) in EtOH (3.00 mL) was added to a round-bottom flask. D-Cys•HCl•H_2O (506 mg, 2.88 mmol) was dissolved in H_2O (3.00 mL). This solution was adjusted to pH 8 using solid potassium carbonate (K_2CO_3) and added to flask containing **2b**. The resulting suspension was stirred at 80 °C for 16 h. The reaction was then cooled and concentrated *in vacuo*. The crude mixture was resuspended in H_2O (10 mL) and washed with EtOAc (2 x 10 mL) to remove unreacted starting material. The aqueous solution was acidified to pH 2 with 1 M sodium bisulfate monohydrate, and the resulting precipitate was filtered to afford **NDMAL** as an orange solid (92 mg, 54%). Spectral data were consistent with literature values.[4] ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ 8.10 (d, *J* = 1.5, 1H), 7.89 (d, *J* = 9.2, 1H), 7.79 (dd, *J* = 8.6, 1.8, 1H), 7.69 (d, *J* = 8.7, 1H), 7.27 (dd, *J* = 9.1, 2.6, 1H), 6.96 (d, *J* = 2.4, 1H), 5.29 (dd, *J* = 9.3, 8.1, 1H), 3.73–3.59 (m, 2H), 3.05 (s, 6H).

(S)-2-(6-(dimethylamino)-naphthalen-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (NPAL)

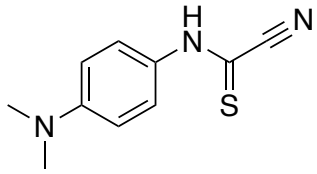


$C_{18}H_{18}N_2O_2S$
MW: 326.41

Following the general procedure of Takakura, *et al*.,[4] compound **2c** (53.6 mg, 0.241 mmol) in EtOH (1.20 mL) was added to a round-bottom flask. D-Cys•HCl•H_2O (218 mg, 1.25 mmol) was dissolved in H_2O (1.20 mL). This solution was adjusted to pH 8 using solid potassium carbonate (K_2CO_3) and added to the flask containing **2c**. The resulting suspension was stirred at 80 °C for 16 h. The reaction was then cooled and concentrated *in vacuo*. The crude mixture was resuspended in H_2O (10 mL) and washed with EtOAc (2 x 10 mL) to remove unreacted starting material. The aqueous solution was acidified to

pH 2 with 1 M sodium bisulfate monohydrate, and the resulting precipitate was filtered to afford **NPAL** as an orange solid (71 mg, 90%). ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 12.83 (s, 1H), 8.07 (d, *J* = 1.8, 1H), 7.88 (d, *J* = 9.0, 1H), 7.77 (dd, *J* = 8.6, 1.9, 1H), 7.65 (d, *J* = 8.7, 1H), 7.08 (dd, *J* = 9.0, 2.4, 1H), 6.78 (d, *J* = 2.3, 1H), 5.27 (dd, *J* = 9.3, 8.1, 1H), 3.73–3.57 (m, 2H), 3.40–3.36 (m, 4H), 2.02–1.99 (m, 4H). ^{13}C NMR (151 MHz, $(CD_3)_2SO$) δ 172.1, 168.3, 147.0, 136.6, 130.0, 129.4, 125.7, 124.8, 124.7, 124.5, 116.6, 104.0, 78.2, 47.4, 34.8, 25.0. HRMS (ESI+) calculated for $C_{18}H_{18}N_2O_2SNa$ $[M+Na]^+$ = 349.0982, found 349.0985.

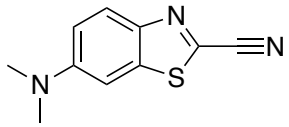
(4-(dimethylamino)phenyl)carbamothioyl cyanide (4)



$C_{10}H_{11}N_3S$
MW: 205.28

Following the general procedure from McCutcheon, *et al.*,[5] dimethyl-4-phenylene diamine (393 mg, 2.9 mmol) in MeCN (14 mL) and THF (14 mL) was added to a flame-dried round bottom flask. 4,5-dichloro-1,2,3-dithiazole (Appel's salt)[6] was added, and the resulting solution was stirred for 10 min at rt. Pyridine (480 μL, 5.8 mmol) was added dropwise to the flask. The mixture was stirred for 60 min (when TLC with 4:6 hexanes:EtOAc indicated full consumption of dimethyl-4-phenylene diamine). A solution of sodium thiosulfate (1.36 g in 7 mL H_2O) was then added, and the mixture was stirred for 1 h at rt. The reaction was poured into a separatory funnel containing 1 M sodium bisulfate (50 mL) and extracted with EtOAc (3 x 50 mL). This solution was washed with 1 M sodium bisulfate (3 x 50 mL) and brine (3 x 50 mL). The organic layers were combined and dried over $MgSO_4$. This mixture was then filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography (eluting with a gradient of 10-30% EtOAc in hexanes) to afford product **4** as a red solid (120 mg, 20%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (m, 2H), 6.73 (m, 2H), 3.01 (s, 6H).

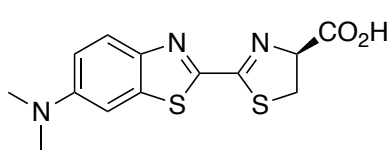
6-(dimethylamino)benzo[*d*]thiazole-2-carbonitrile (5)



$C_{10}H_9N_3S$
MW: 203.26

Following the general procedure from McCutcheon, *et al.*,[5] palladium chloride ($PdCl_2$, 12 mg, 0.06 mmol), copper iodide (CuI, 60 mg, 0.3 mmol) and tetrabutyl ammonium bromide (TBAB, 373 mg, 1.2 mmol) in DMF (9.5 mL) were added to a flame-dried round bottom flask. **4** (120 mg, 0.6 mmol) in DMSO (9.5 mL) was added. The resulting suspension was stirred for 1 h at 130 °C. The reaction was then cooled, diluted with EtOAc (30 mL), and washed with H_2O (3 x 30 mL) and brine (3 x 30 mL). The organic layers were combined and dried over $MgSO_4$. This mixture was then filtered and concentrated *in vacuo.* The crude product was purified by flash column chromatography (eluting with 20% acetone in hexanes) to afford product **5** as a yellow solid (37 mg, 31%). ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 7.93 (dd, J = 9.3, 0.9, 1H), 7.30 (t, J = 2.2, 1H), 7.18 (ddd, J = 9.3, 2.7, 1.1, 1H), 3.10 (s, 6H).

Potassium (*S*)-2-(6-(dimethylamino)benzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylate (6′Me$_2$N-Luc)



$C_{13}H_{12}KN_3O_2S_2$
MW: 345.48

Following the general procedure from McCutcheon, *et al.*,[5] compound **5** (37 mg, 0.18 mmol) and D-Cys•HCl•H_2O (45 mg, 0.20 mmol) in MeCN (2 mL) were added to a round-bottom flask. K_2CO_3 (25 mg, 0.18 mmol) in H_2O (180 µL) was added. The resulting suspension was stirred at 2.5 h. The reaction was filtered and solids were dried. The crude material was triturated with wetted MeCN and filtered to afford **6′Me$_2$N-Luc** as an orange

potassium salt (25 mg, 39%). ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.85 (d, J = 9.1, 1H), 7.27 (d, J = 2.6, 1H), 7.01 (dd, J = 9.2, 2.6, 3H), 4.94 (dd, J = 9.5, 8.3, 1H), 3.70–3.43 (m, 2H), 2.98 (s, 6H).

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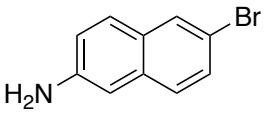
3. Gammon, S. T.; Leevy, W. M.; Gross, S.; Gokel, G. W.; Piwnica-Worms, D. *Anal. Chem.* **2006**, *78*, 1520–1527.
4. Takakura, H.; Sasakura, K.; Ueno, T.; Urano, Y.; Terai, T.; Hanaoka, K.; Tsuboi, T.; Nagano, T. *Chem. Asian. J.* **2010**, *5*, 2053–2061.
5. McCutcheon, D. C.; Porterfield, W. B.; Prescher, J. A. *Org. Biomol. Chem.* **2015**, *13*, 2117–2121.
6. Appel, R.; Janssen, H.; Siray, M.; Knock, F. *Chem. Ber.* **1985**, *118*, 1632–1643.

IV. NMR Spectra



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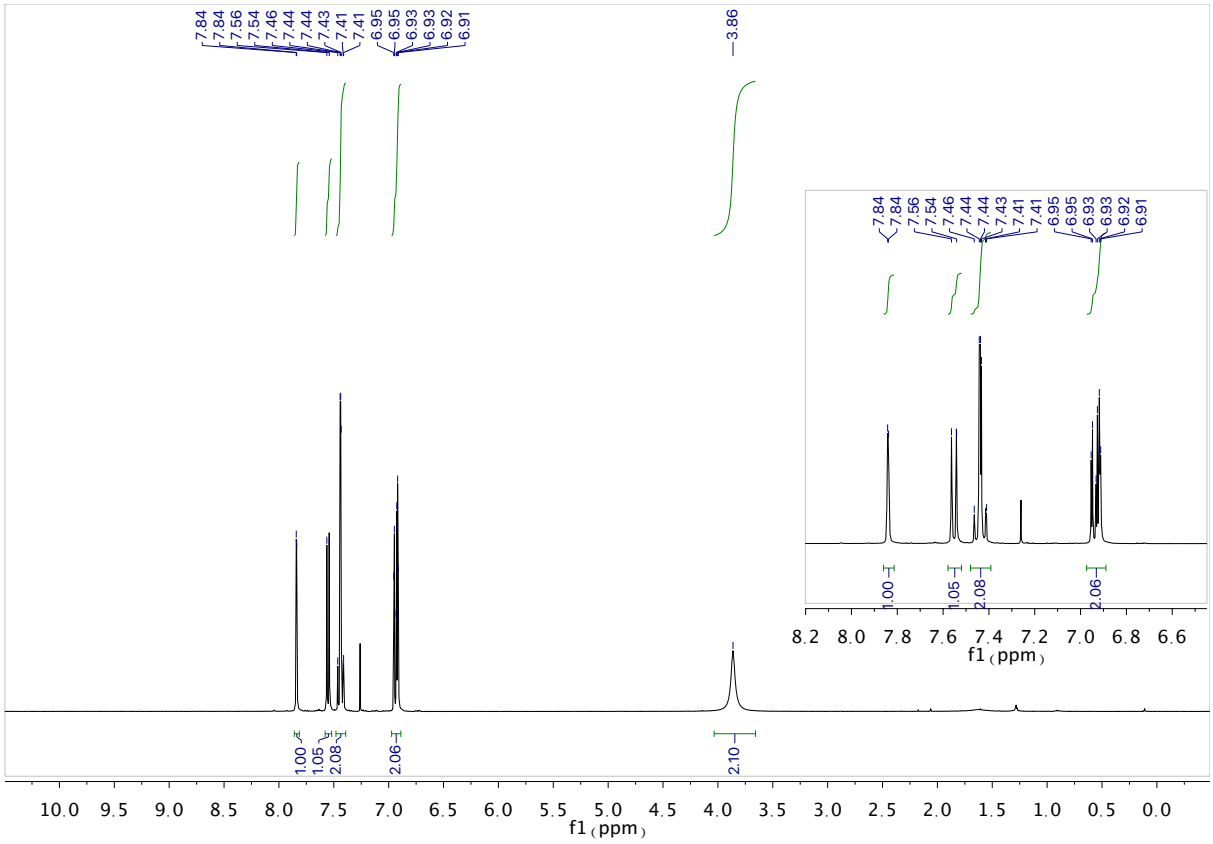

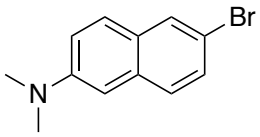
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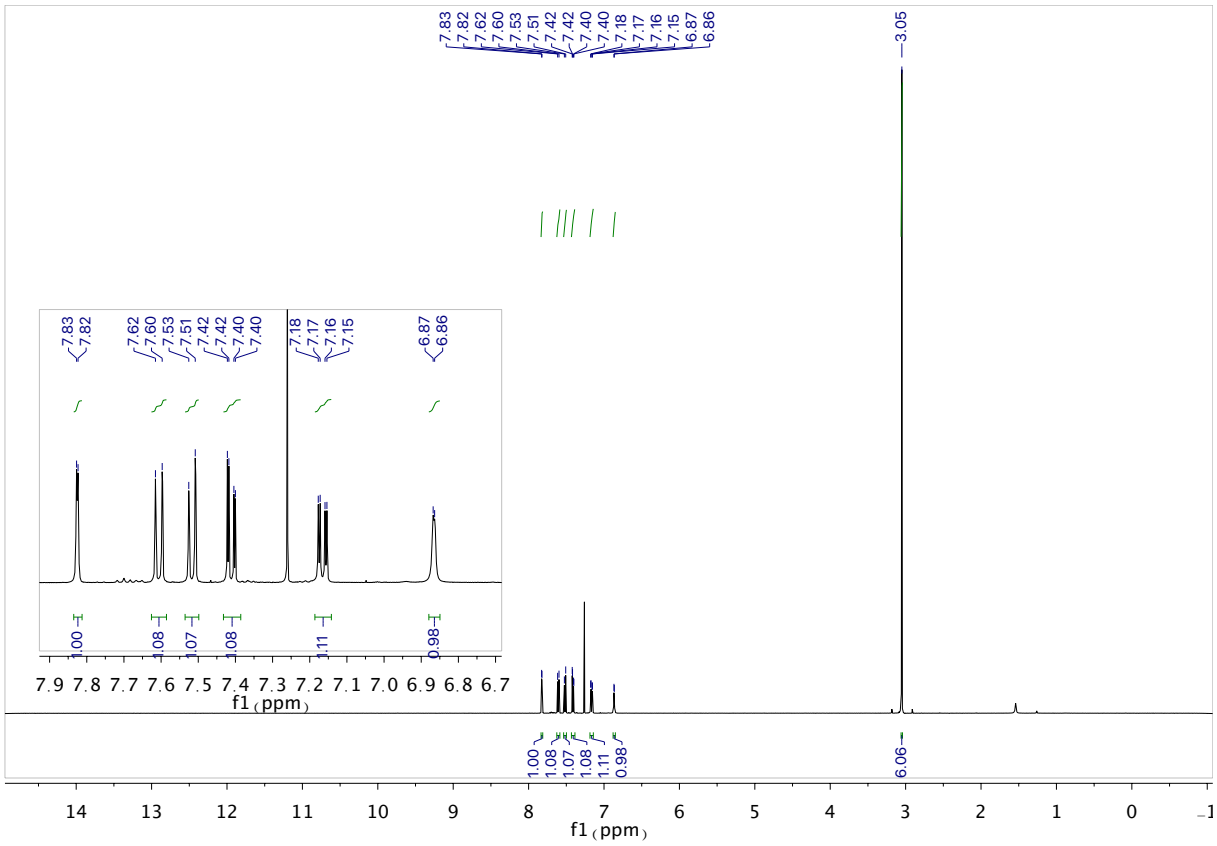
N

1b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1.08
1.07
1.08
1.11
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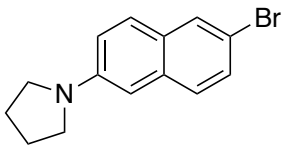
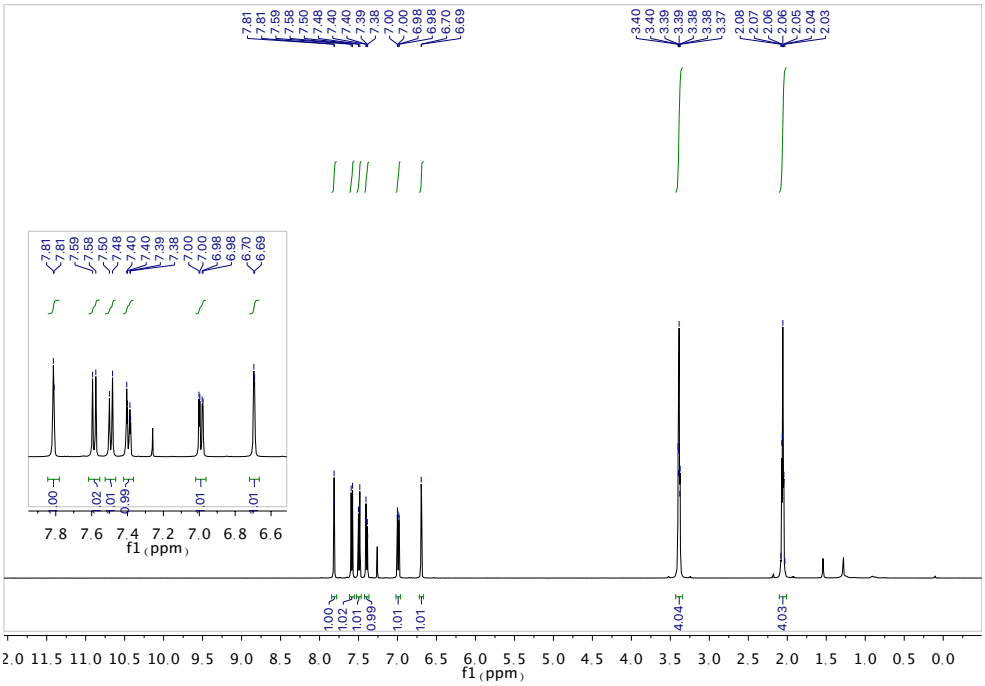
6.06
f1 (ppm)


Br
N

1c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3.38
3.37
2.08
2.07
2.06
2.06
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1.01
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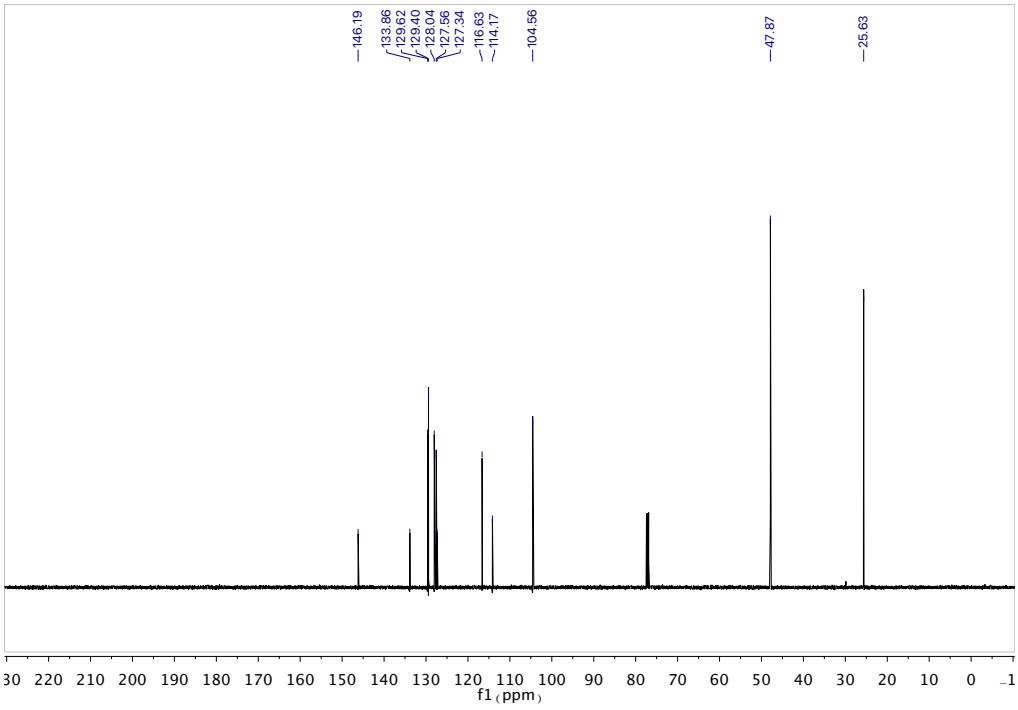
f1 (ppm)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127.34
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104.56
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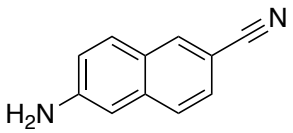
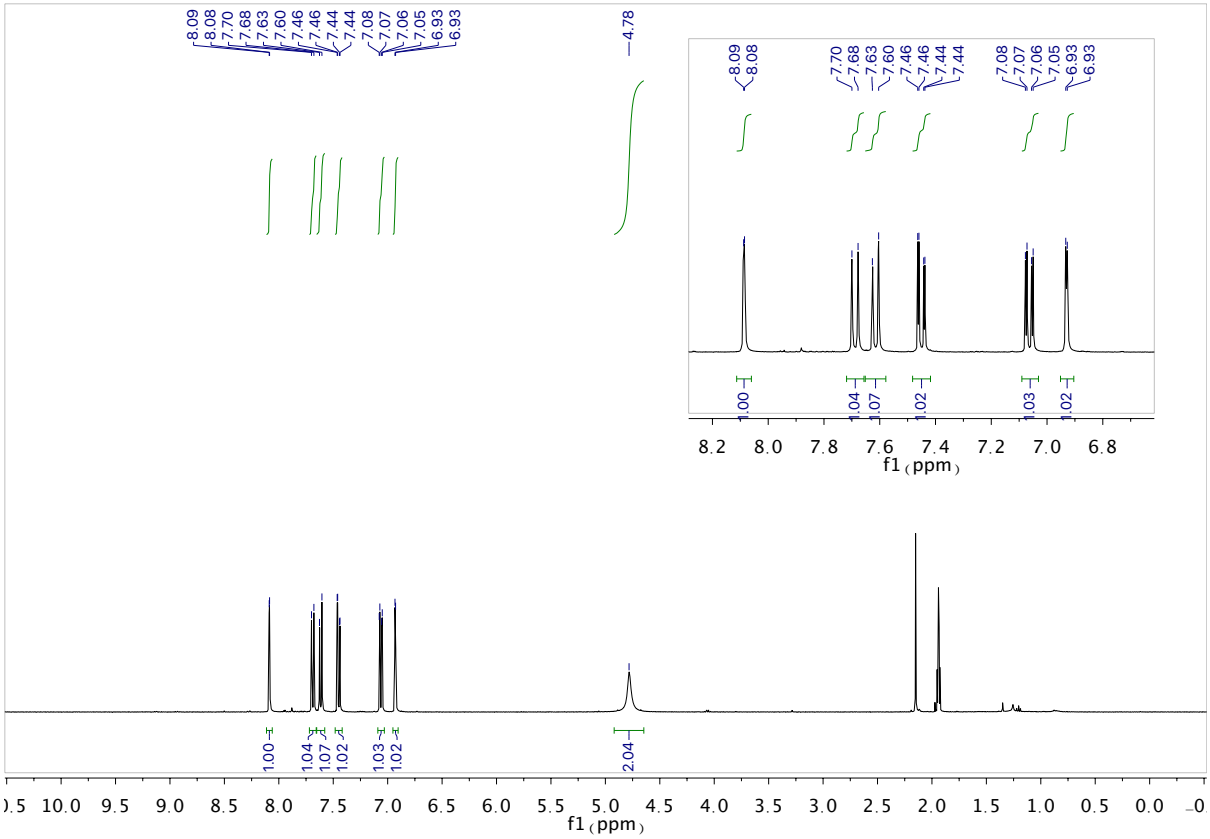
25.63
f1 (ppm)


N
H2N

2a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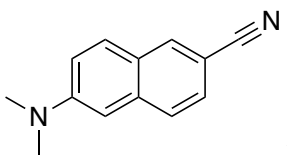
1.03
1.02
2.04
f1 (ppm)



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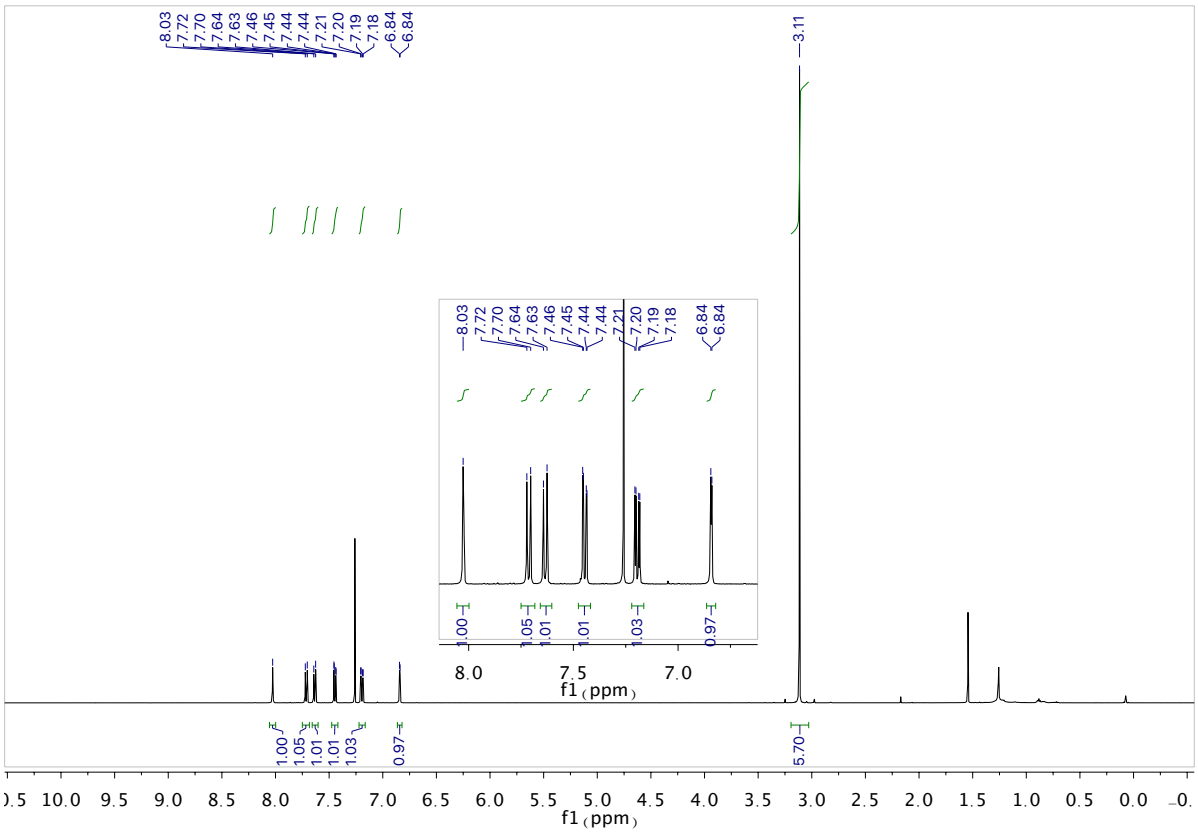
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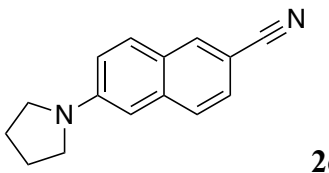
f1 (ppm)



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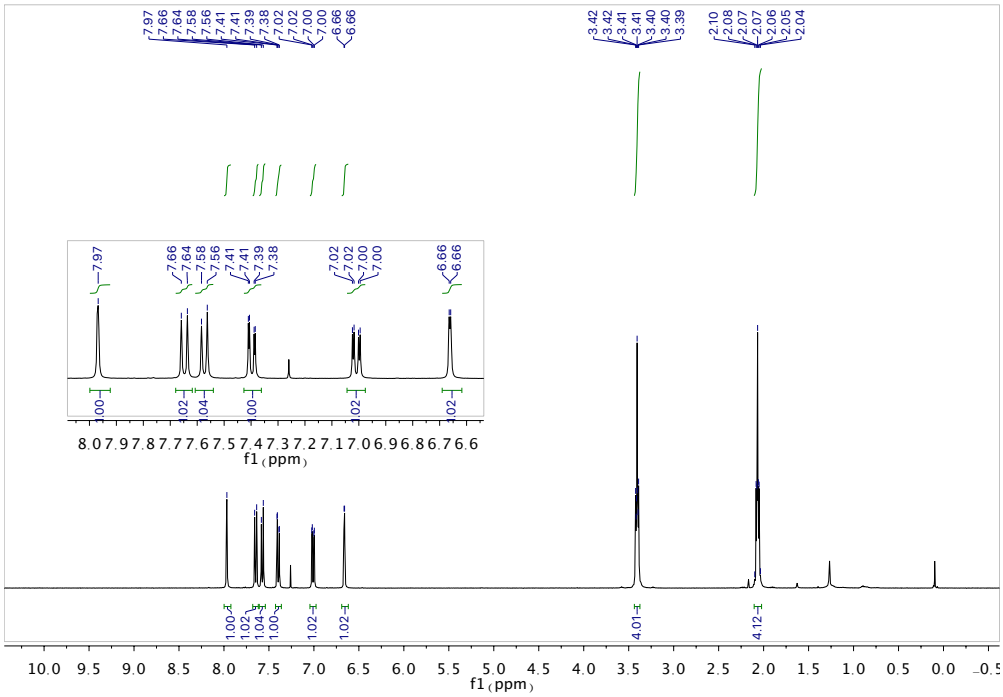
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3.40
3.40
3.39
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2.08
2.07
2.07
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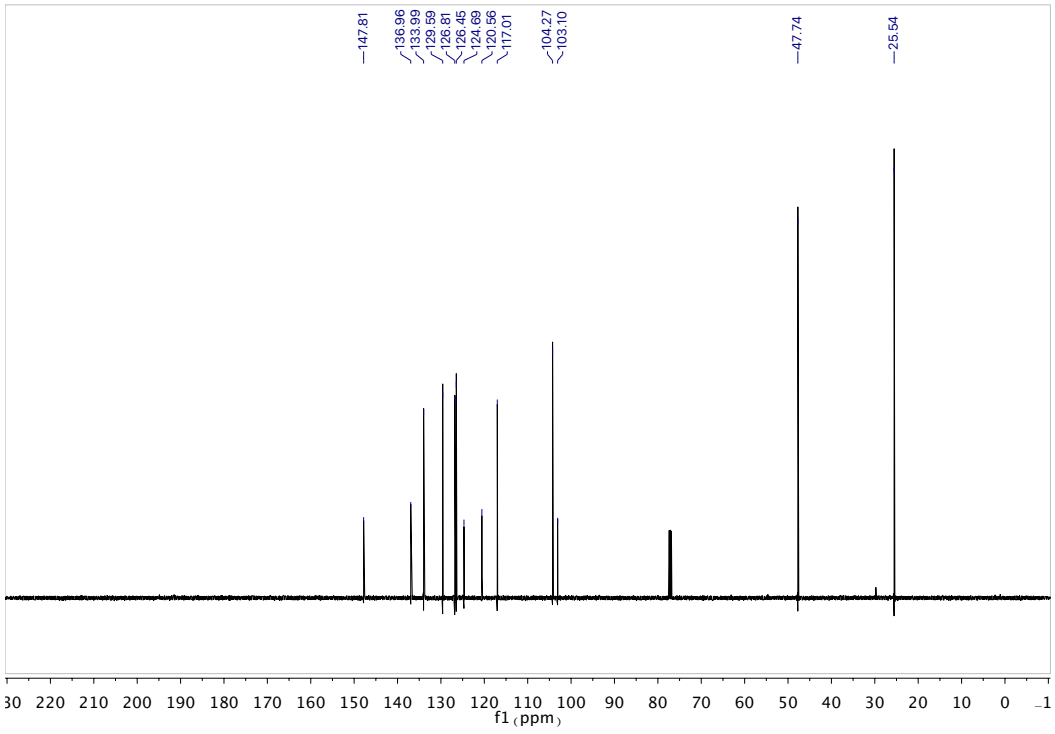
f1 (ppm)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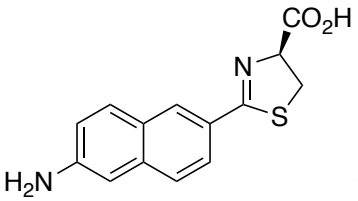
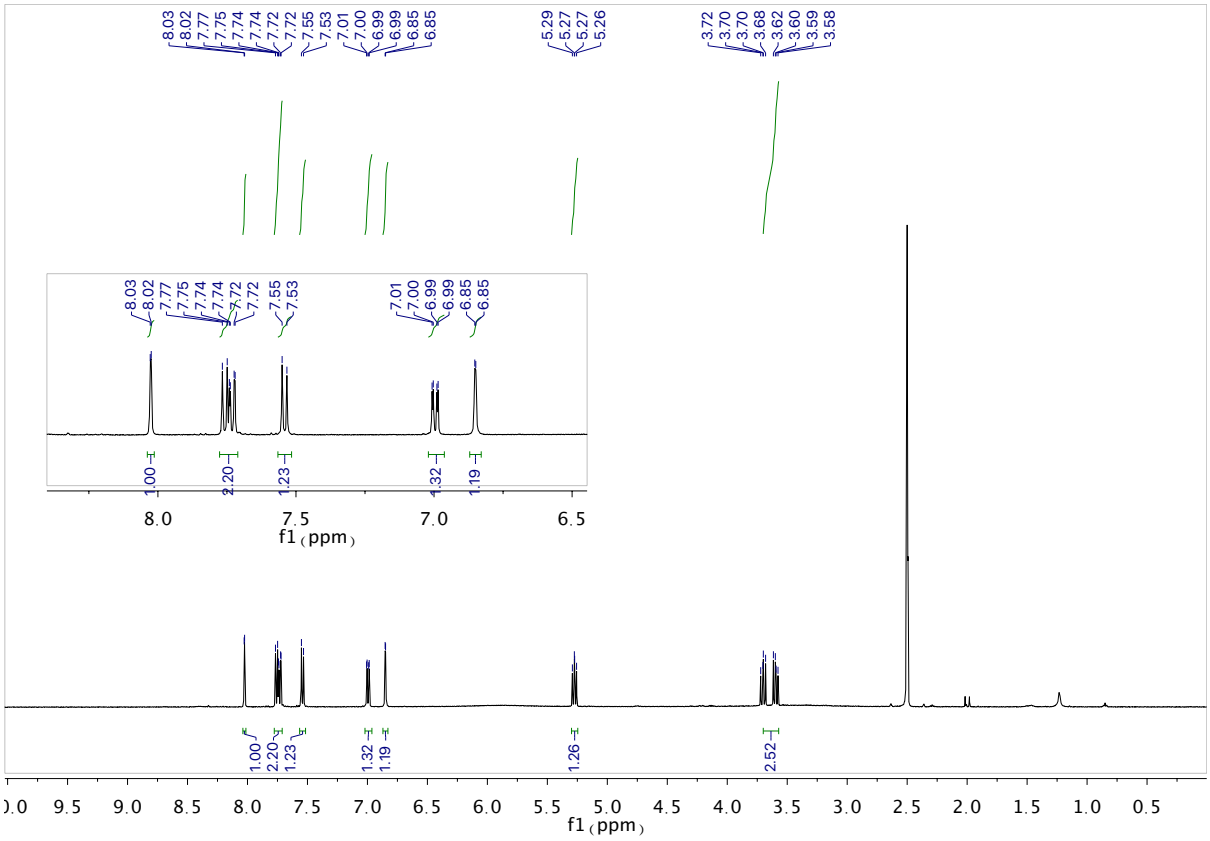
f1 (ppm)


CO2H
N
S
H2N

NAL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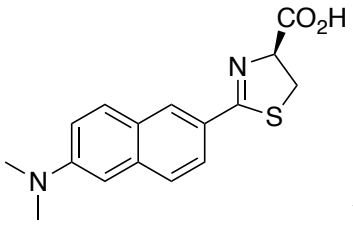
f1 (ppm)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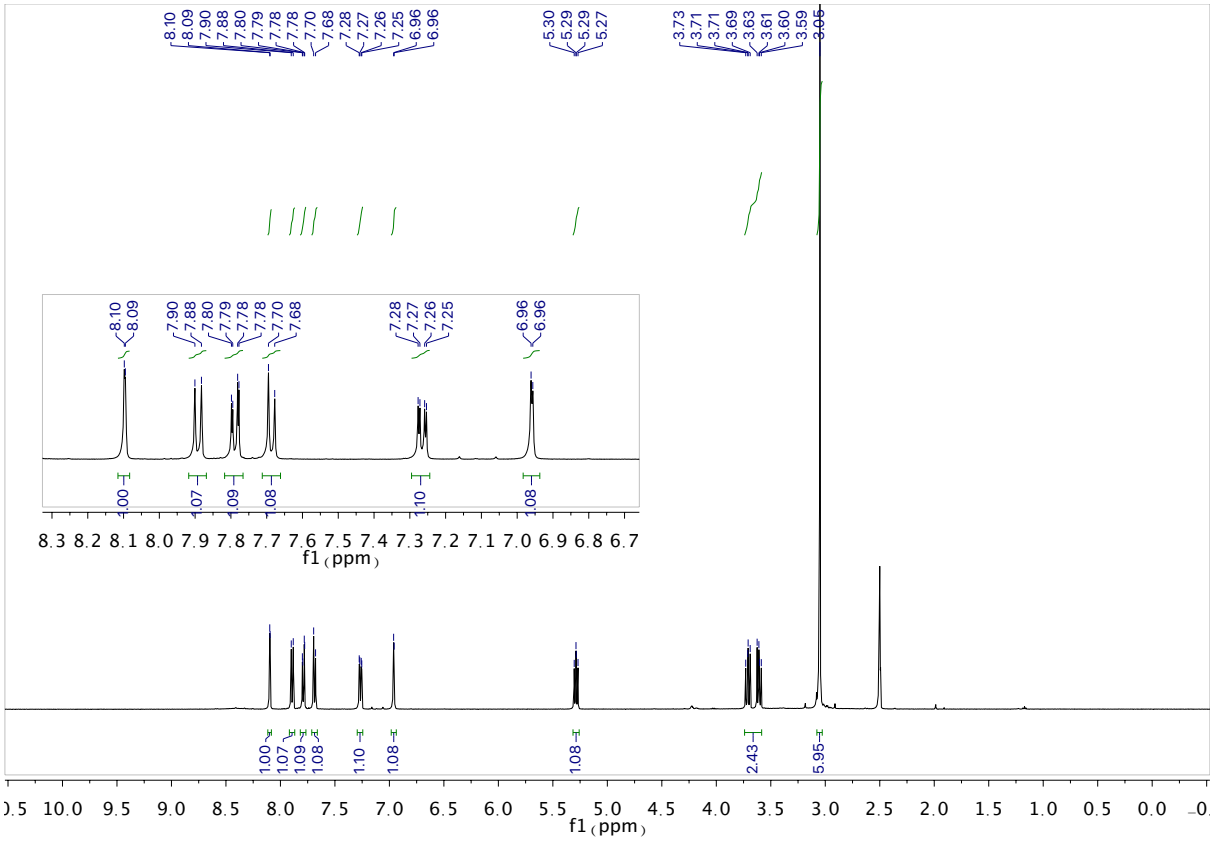
S
N

NDMAL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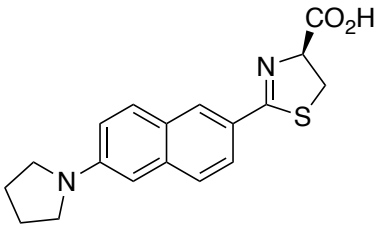
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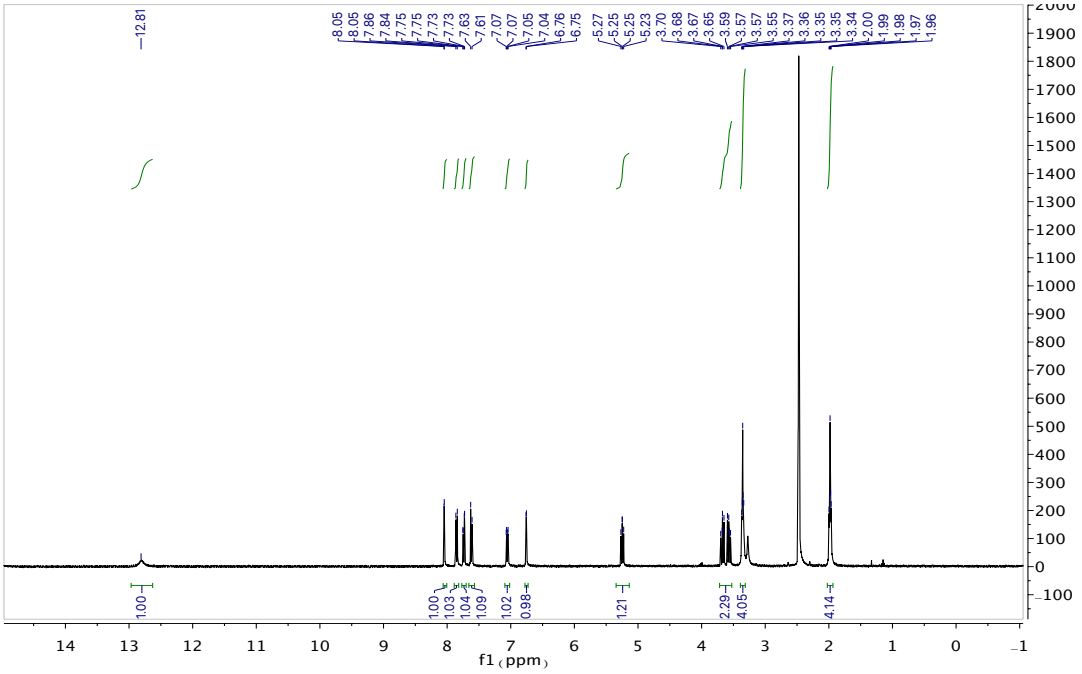
S
N

NPAL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6.75
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5.25
5.25
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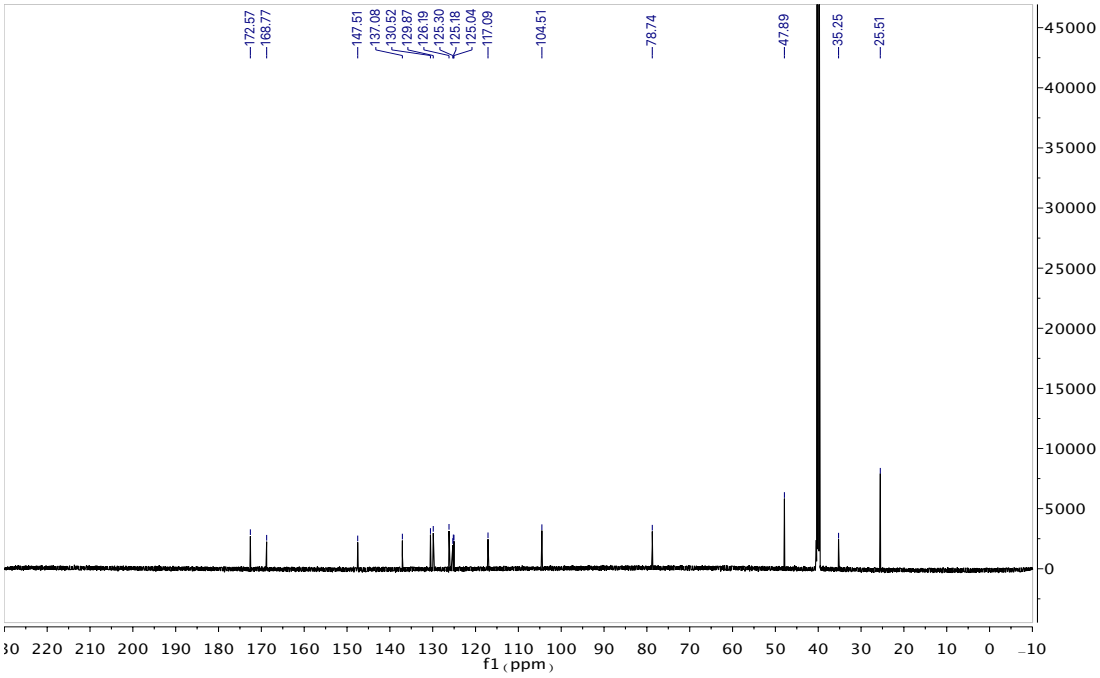
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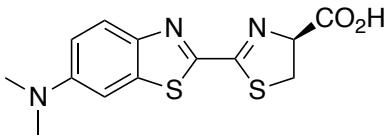
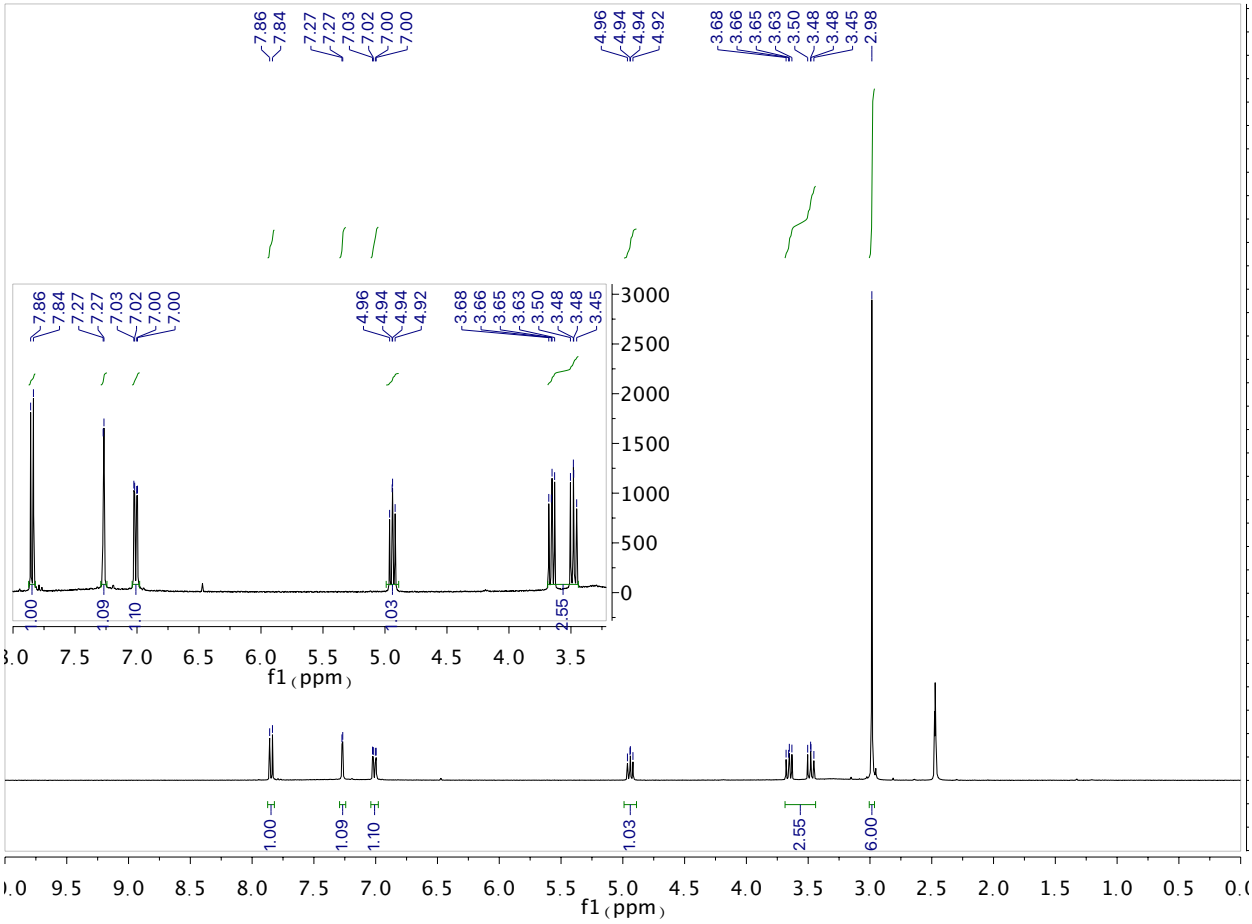

N
N
CO2H
S
S
N

6′Me$_2$N-Luc


7.86
7.84
7.27
7.27
7.03
7.02
7.00
7.00
4.96
4.94
4.94
4.92
3.68
3.66
3.65
3.63
3.50
3.48
3.48
3.45
2.98
1.00
1.09
1.10
1.03
2.55
6.00
f1 (ppm)