Supporting Information for

A versatile bioluminescent probe with tunable color

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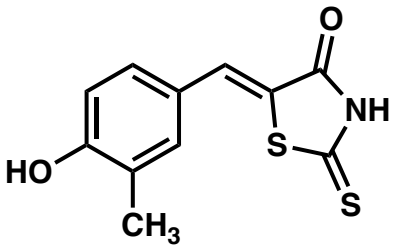
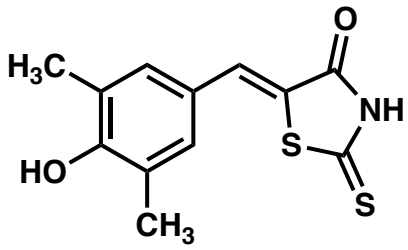
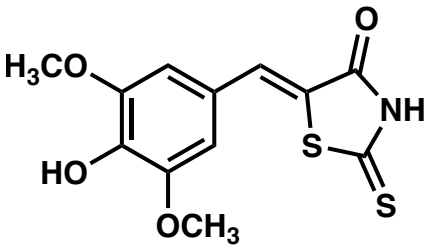
Table of contents



HMBR



HBR-3,5DM



HBR-3,5DOM

Fluorogen	λ ex (nm)	λ em (nm)
HMBR	481	542
HBR-3,5DM	501	561
HBR-3,5DOM	520	600

Figure S1. Fluorogenic ligands evaluated in this study. The excitation and emission wavelengths are from Benaissa, *et al*.[1]

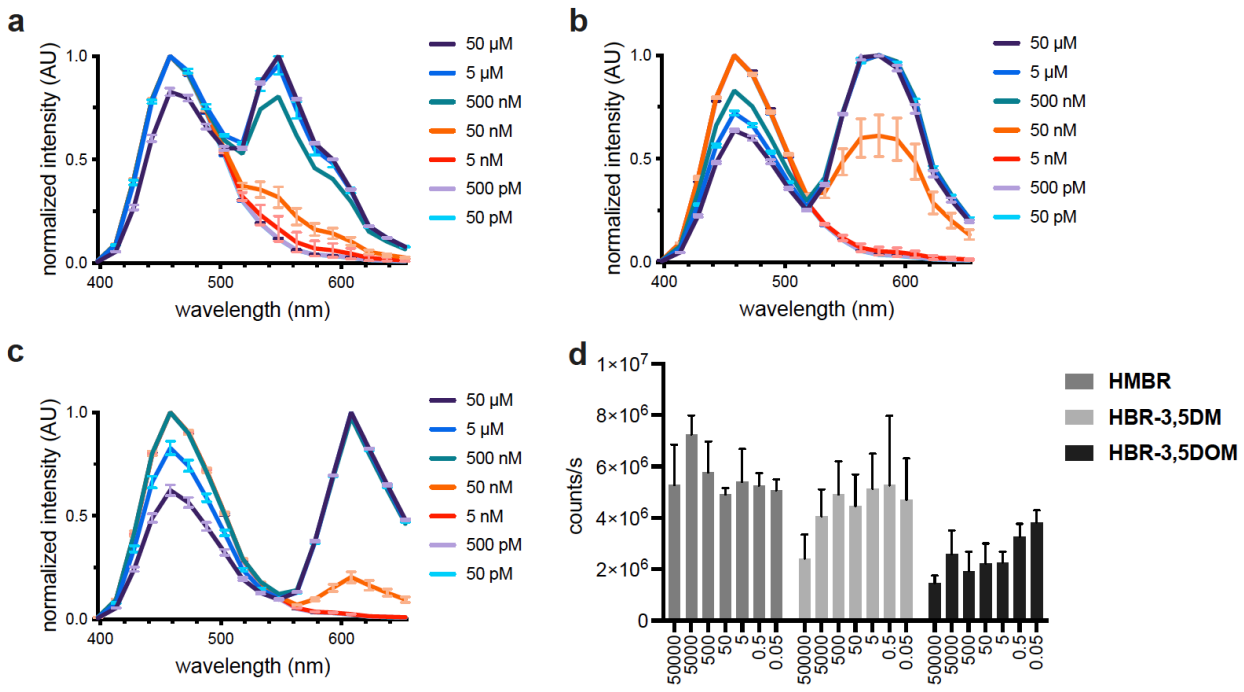
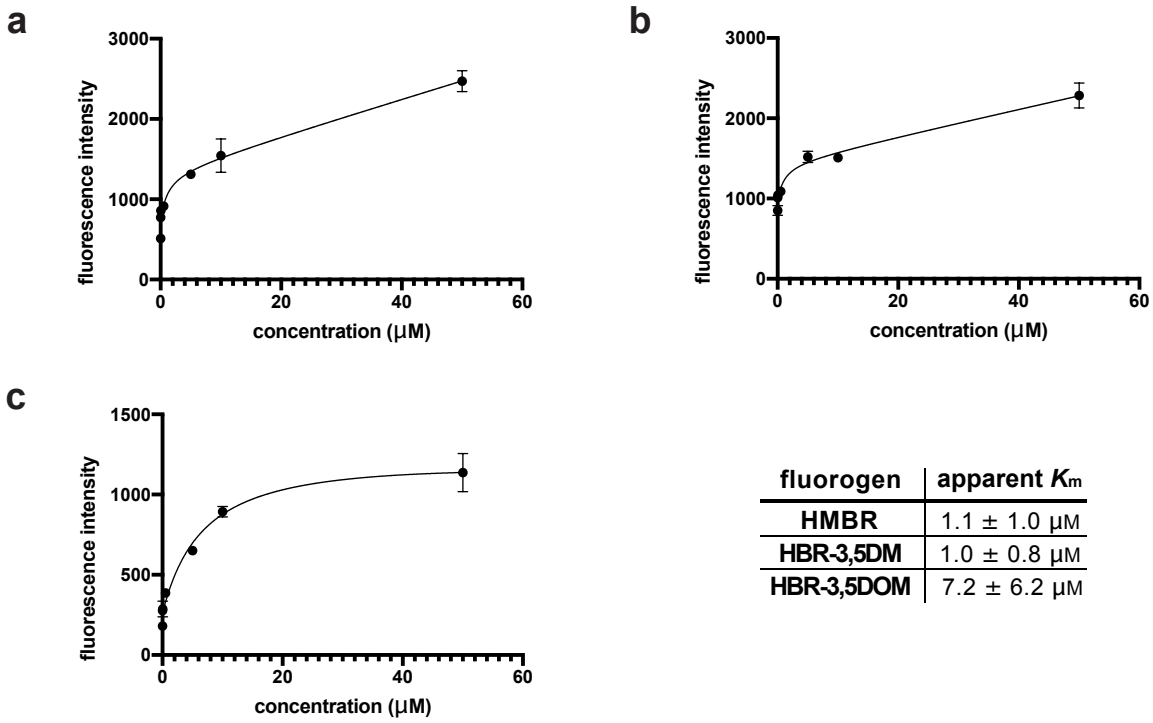
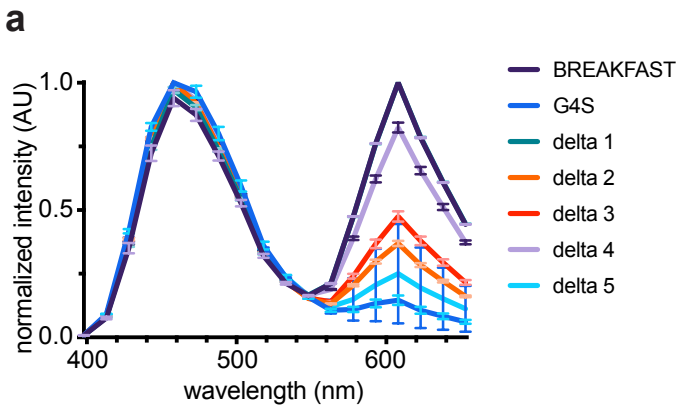


Figure S2. Bioluminescence emission spectra vary with ligand concentration. HeLa cells stably expressing BREAKFAST were treated with Fz (20 µM or 1:100 of commercial stocks) and the indicated concentrations of pFAST ligand (a) **HMBR**, (b) **HBR-3,5DM**, or (c) **HBR-3,5DOM**. Spectra were acquired from 400–650 nm. (d) Total photon output (counts per second) for samples in the presence of Fz and varying concentrations (0.05–50,000 nM) for **HMBR** (dark gray bars), **HBR-3,5DM** (light gray bars), or **HBR-3,5DOM** (black bars). Error bars represent the standard error of the mean for $n = 3$ experiments.



fluorogen	apparent K_m
HMBR	1.1 ± 1.0 μM
HBR-3,5DM	1.0 ± 0.8 μM
HBR-3,5DOM	7.2 ± 6.2 μM

Figure S3. Binding assays with selected fluorogens. Reaction profiles of (a) **HMBR**, (b) **HBR-3,5DM**, or (c) **HBR-3,5DOM**. Binding affinities are tabulated as apparent values, determined via measurements of light emission over a range of substrate concentrations. Error bars represent the standard error of the mean for $n = 3$ experiments.



b

fusion	BRET efficiency
BREAKFAST	0.49
NlucΔ1	0.45
NlucΔ2	0.24
NlucΔ3	0.30
NlucΔ4	0.41
NlucΔ5	0.13
1xG4S	0.14

Figure S4. Bioluminescence emission spectra of BREAKFAST probes comprising alternative linkages. Amino acids (1-5) were removed from the N-terminus of NanoLuc. (a) Bioluminescence spectra of the five deletion constructs (delta 1-5), a construct comprising a G_4S linker between NanoLuc and pFAST, and the initial BREAKFAST construct. Spectra were acquired in the presence of Fz (20 μM or 1:100 of commercial stocks) and **HBR-3,5DOM** (20 μM). Error bars represent the standard error of the mean for $n = 3$ experiments. b) Tabulated values for BRET efficiencies from the outputs measured in (a), shown as the mean value from $n = 3$ experiments.

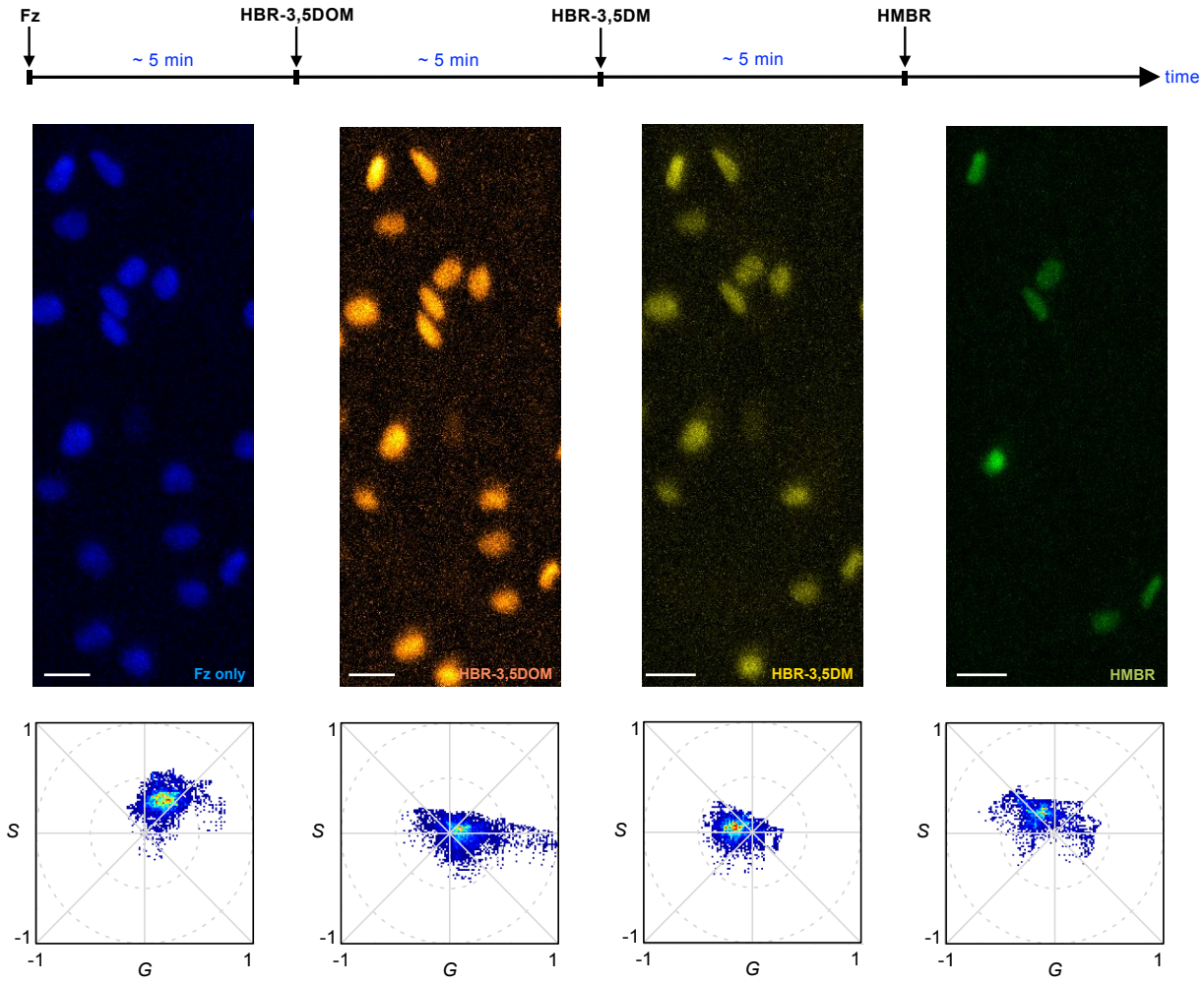


Figure S5. Color tuning with BREAKFAST probes. Wide field of view of data set from Figure 4. A549 cells were engineered to express NanoLuc-pFAST in the nucleus. Luciferin (Fz) and fluorogens were then flowed in as depicted, and images were acquired between each addition. Distinct spectral outputs were observed depending on the ligand present. Some cells detached in the imaging chamber under flow. Scale bars = 10 μm

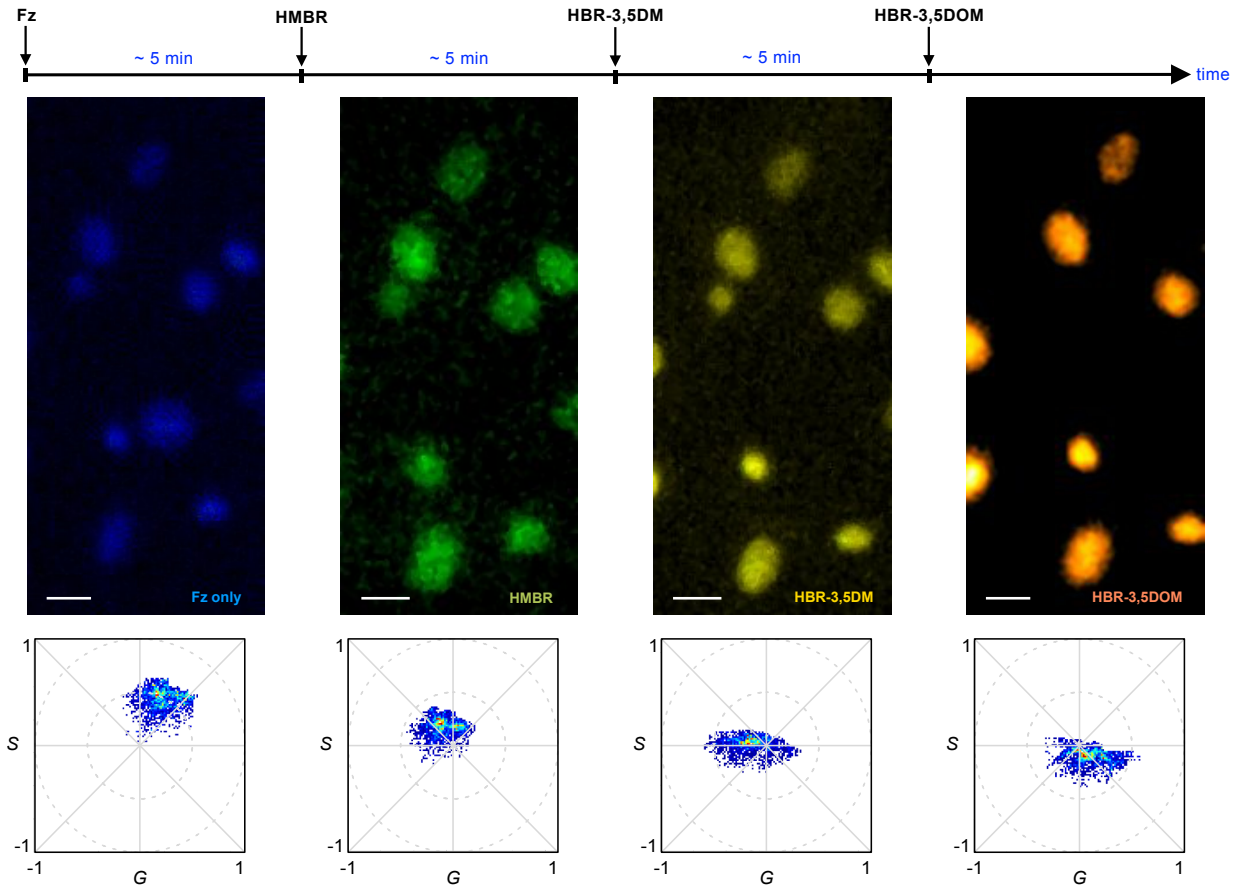


Figure S6. Reverse color tuning with BREAKFAST probes. A549 cells were engineered to express NanoLuc-pFAST in the nucleus. Luciferin (Fz) and fluorogens were added in the reverse order from Figures 4 and S5 (as shown). Images were acquired between each addition. Distinct spectral outputs were observed depending on the ligand present. Some cells detached in the imaging chamber under flow. Scale bars = 10 μm

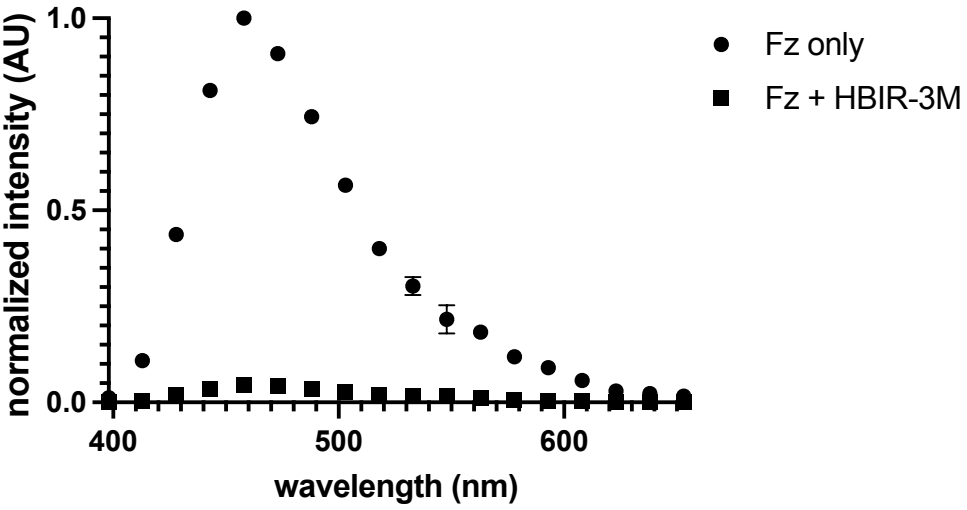


Figure S7. Bioluminescence quenching with **HBIR-3M**. Purified BREAKFAST (200 nM) was treated with Fz (20 μM or 1:100 of commercial stocks) only or in the presence of **HBIR-3M** (50 μM). Bioluminescence emission spectra were then acquired.

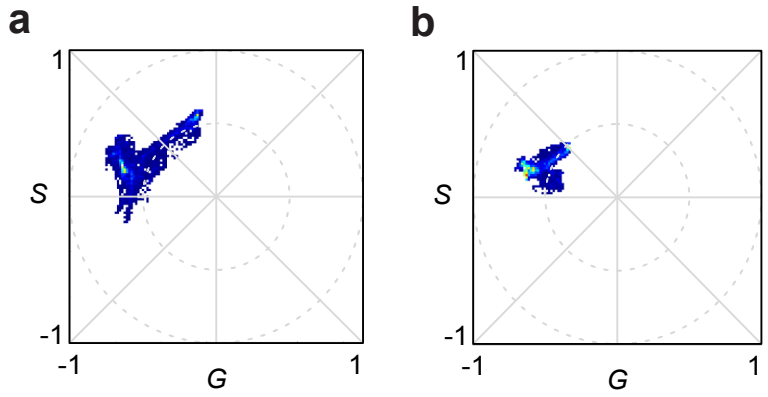


Figure S8. Phasor spectra for reversible bioluminescence quenching. Bioluminescence images from Figure 5 are shown from samples (a) prior to quenching and (b) following of **HMBR** treatment.

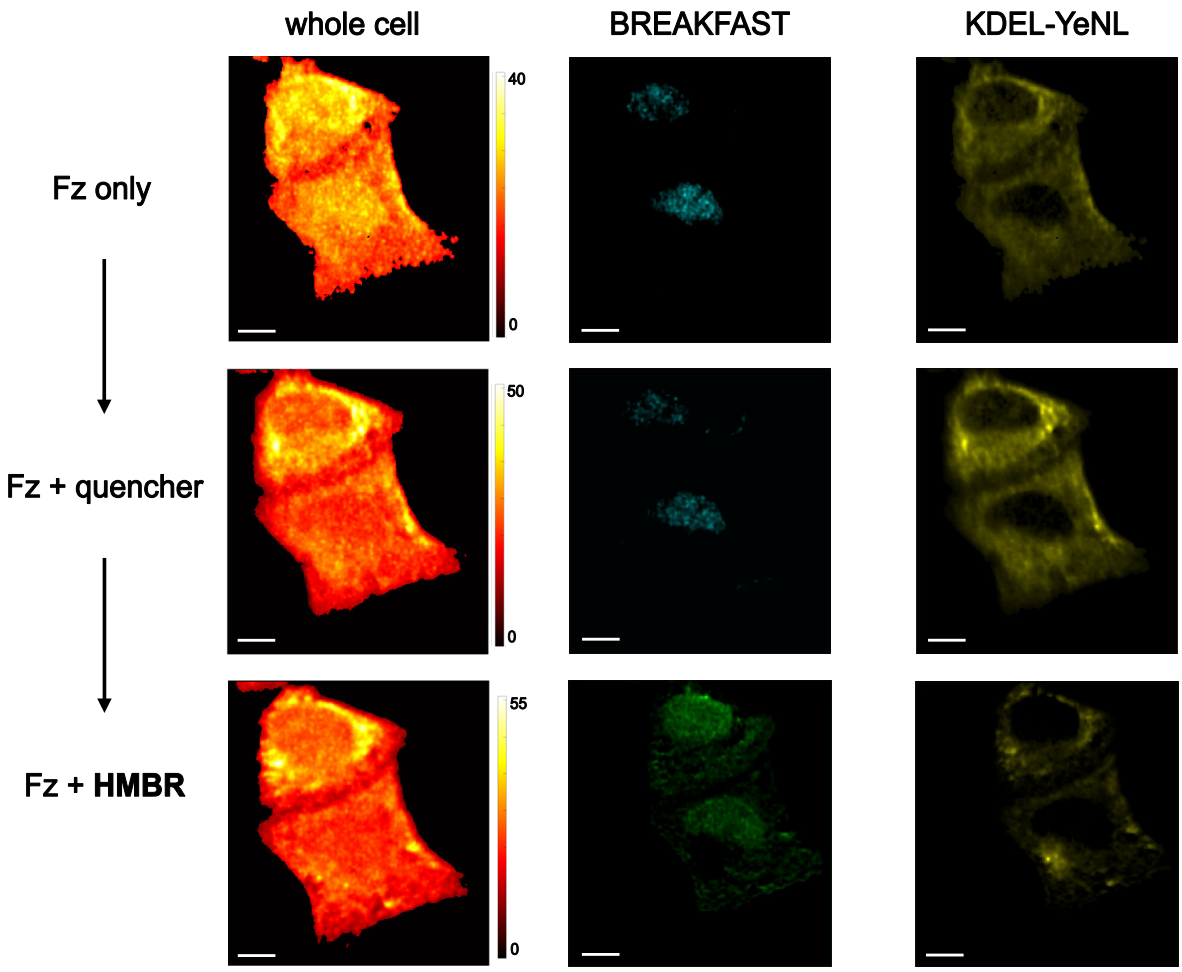


Figure S9. Additional example of bioluminescence quenching. HeLa cells were transiently co-transfected with KDEL-YeNL and nuclear localized BREAKFAST reporters. Cells were treated with Fz only, followed by **HBIR-3M** (quencher), and finally **HMBR** (fluorogen). Signal from BREAKFAST and YeNL were unmixed. Image scale bars = 10 μm; vertical scale bars are measures of intensity (in a.u.).

Primer lists

All primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA) and are written in the 5′ → 3′ direction.

Table S1. Primers used to assemble plasmids.

primer name	sequence
ZT_017_F	gtttaactttaagaaggagatatacatatggagcatgttgccttt
ZT_21_R	gagtgtgaagaccatagagcctccgccgcccacccgtttcacaaa
ZT_036_R	ccagtccccaacgaaatcttccacccgtttcacaaagac
ZT_037_R	gtccccaacgaaatcttcgagcacccgtttcacaaagac
ZT_038_F	atcaccatcaccatcacggatccatggagcatgttgcct
ZT_39_R	gcctttcgttttatttgatgcctctagattacgccagaatgcgttc
ZT_040_F	cactatagggagacccaagcttatggagcatgttgccttt
ZT_041_R	acactatagaatagggccctctagattacgccagaatgcg
ZR_042_R	aacgaaatcttcgagtgtgaacacccgtttcacaaagac
ZT_043_R	aacgaaatcttcgagtgtgaacacccgtttcacaaagac
ZT_044_R	gaaatcttcgagtgtgaagaccacccgtttcacaaagac
ZT_054_F	tctttgtgaaacgggtgatggtcttcacact
ZT_055_R	gtttttgttcgtcgaccgccagaatgcgttc
ZT_057_F	gaacgcattctggcggaattcagcagggct
ZT_058_R	atagggccctctagactcgagtcacacctt

Methods and materials

Reagents

All reagents purchased from commercial suppliers were of analytical grade and used without further purification. All fluorogenic ligands were prepared according to previously published procedures and spectra matched reported data.[1,2] Relevant NMR spectra are included below.

General biological materials

Unless otherwise specified, materials (buffer salts, dNTPs, etc.) were purchased from Fisher Scientific. Chemically competent *E. coli* cells (TOP10 or BL21) were used for transformations. Bacteria were grown in Luria-Bertani (LB) media containing 1X ampicillin (AMP, 100 μg/mL). Furimazine (Fz) was purchased from Promega as Nano-Glo luciferase assay substrate and used at either a 1:100 or 1:50 dilution. The plasmid encoding for the sequence for pFAST was purchased from Addgene. The protein encoding sequence for NanoLuc was purchased from Twist Bioscience.

General cloning methods

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) T_m 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 pcDNA3.1+ vectors were generated via digestion with restriction enzymes (New England BioLabs). The pFAST-NanoLuc fusion was prepared using the pFAST sequence (derived from Addgene plasmid #172861) and the NanoLuc sequence (prepared by Twist Bioscience). All other pFAST-NanoLuc constructs were prepared using the primers in Table S1 to amplify desired BREAKFAST inserts. For these constructs, linearized vectors were combined with the appropriate BREAKFAST insert by Gibson assembly (50 °C for 60 min). A portion of the reactions (3.0 μL) was directly transformed into TOP10 competent *E. coli* cells. Colonies containing the genes of interest were expanded overnight in 5 mL LB broth supplemented with ampicillin (100 μg/mL) or kanamycin (100 μg/mL) and DNA was extracted from colonies using a Zymo Research Plasmid Mini-prep Kit. Sequencing analysis confirmed successful plasmid generation (Genewiz).

General bioluminescence imaging

All bioluminescence assays were performed in black 96-well plates (Greiner Bio One). Plates were imaged with an IVIS Lumina (Xenogen) equipped with a CCD camera chilled to –90 °C or a Tecan F200 Pro injection port luminometer with a neutral density filter. The stage for the IVIS Lumina was kept at 37 °C during each imaging session, and the camera was controlled using Living Image software. For assays with bacterial cells, the exposure times ranged from 1–10 s. Data binning levels were set to medium for all imaging reads. Regions of interest were selected for quantification and total flux values were analyzed using Living Image software. Measurements were acquired in either duplicate or triplicate, and the data were analyzed using Microsoft Excel or GraphPad Prism (version 9.0 for Macintosh, GraphPad Software).

Photophysical measurements

Thermodynamic dissociation constants were determined via titration experiments. Fluorescence measurements were obtained with various ligand concentrations in the presence of purified BREAKAST or probe in bacterial lysate. Measurements were performed on a Tecan F200 Pro injection port luminometer using a constant protein concentration. Data were fit using Prism 9 software with a one-site specific binding model. Bret efficiencies were calculated using area under the curve (AUC) measurements.

Mammalian cell culture

HeLa (ATCC), HEK293 (ATCC), A549 (ATCC), and MDA-MB231 cells (ATCC) were cultured in complete medium: DMEM (Corning) containing 4.5 g/L glucose, 2 mM L-glutamine, and

supplemented with 10% (v/v) FBS (Life Technologies), penicillin (100 U/mL) and streptomycin (100 µg/mL, Gibco). Cells were incubated at 37 °C in a 5% CO_2 humidified chamber and serially passaged using trypsin (0.25% in HBSS, Gibco). Cells were counted using an automated cell counter (Countess II, Invitrogen).

Transient transfections

Cells (2.5×10^5) were seeded in 12-well dishes (Corning). Transfections were performed with luciferase constructs using Lipofectamine 2000 or 3000 (ThermoFisher) according to the manufacturer's instructions. Transfections were performed when cells were 75–80% confluent (1 d post seeding). Approximately 16 h post-transfection, cells were lifted with trypsin and plated (5×10^5 cells) in tissue-culture-treated eight-well chambered coverslips (µ-Slide 8 Well ibiTreat, Ibidi). The coverslips were coated with 5 mg/cm^2 fibronectin human plasma (Millipore Sigma) according to the manufacturer's instructions.

Viral transductions

BREAKFAST-pLenti, pDisplayBREAKFAST-pLenti, and NuclearBREAKFAST-pLenti plasmids were cloned as described in the General cloning methods section above. The psPAX2 packaging plasmid (Addgene plasmid no. 12260) and pMD2.G envelope-expressing plasmid (Addgene plasmid no. 12259) were gifted from D. Trono. Each plasmid was transformed into chemically competent *E.coli* cells (TOP10). The transformants were plated on agar plates containing carbenicillin (50 µg/mL). Colonies containing the genes of interest were expanded overnight in 5 mL of LB broth containing ampicillin (50 µg/mL). Plasmid DNA was extracted using a Zymo Research Plasmid Mini-prep Kit and concentrations were measured using a

Nanodrop 2000c Spectrophotometer (ThermoScientific). HEK293 cells (2.5×10^5) were seeded in six-well dishes (Corning). BREAKFAST-pLenti, pDisplayBREAKFAST-pLenti, or NuclearBREAKFAST-pLenti plasmids were cotransfected with psPAX2 and pMD2.G using Lipofectamine 3000 (ThermoFisher) according to the manufacturer's instructions. Transfections were performed when cells were 60–75% confluent (1 d post seeding). Approximately 16 h post-transfection, cell media was replaced with fresh complete media supplemented with 10 mM sodium butyrate, 20 mM HEPES and 2 mM L-glutamine. The next day, media was replaced again with fresh complete media. Approximately 48 h post incubation, cell media containing virus was collected and filtered through a 0.45-μm PVDF membrane. Virus was either stored at −80 °C or used immediately for transduction. Transduced cells were further cultured with puromycin (20 μg/mL) to preserve gene incorporation.

Luciferase expression and purification

The pQE-BREAKFAST plasmid was generated using the general cloning methods mentioned above. Plasmids were transformed into chemically competent *E. coli* cells (BL21). The transformants were plated on agar plates containing carbenicillin (carb, 50 μg/mL). Cells were expanded in LB medium containing ampicillin (LB-Amp) at 37 °C overnight. The overnight culture (20 mL) was used to inoculate 1 L of LB-Amp. The new culture was incubated at 37 °C to mid-log phase (optical density ~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. Cells were collected at 4 °C by centrifugation at 4000 x *g* for 15 min. Cell pellets were resuspended in 40 mL of phosphate buffer (35 mM phosphate, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Lysozyme (1 mg/mL final concentration) was added, and the cells were

sonicated and centrifuged at 10,000 x *g* for 1 h at 4 °C. BREAKFAST was purified from clarified supernatants using nickel affinity chromatography (BioLogic Duo Flow Chromatography System, Bio-Rad). The collected fractions were dialyzed into a phosphate buffer (50 mM phosphate, pH 7.5). Dithiothreitol (1 mM final concentration) and 15% glycerol were added to the dialyzed samples prior to aliquoting and storage at −20 °C. Final protein concentrations were determined using A_{280} measurements performed on a JASCO V730 UV-vis spectrophotometer. SDS–PAGE analyses were also performed to verify protein purity. Gels were stained with Coomassie R-250.

Fluorescence microscopy

Fluorescence imaging was performed with a Zeiss LSM880 confocal microscope equipped with a 34-Channel Quasar Detection unit, consisting of a calibrated 32-element gallium arsenide phosphide (GaAsP) array of photomultiplier tubes (PMTs, two side-flanking PMTs in addition to the 32-element PMT array) in photon counting mode and equipped with a ×63/1.4 Oil DIC M27 objective (Zeiss). The excitation wavelength was adapted to the acceptor protein: 488 nm for **HMBR** and **HBR-3,5DM**; and 514 nm for **HBR-3,5DOM**. Pixel size was 0.13 μm, image size was 1024x1024 pixels and acquired with a pixel dwell time of 2.05 μs. The final image was the sum of 4 frames.

Bioluminescence microscopy

Bioluminescent phasors were acquired on an Olympus IX83 Total internal reflection fluorescence (TIRF) microscope equipped with two Optosplit II (Cairn) image splitters and used in widefield mode. The light path is described in Yao, *et al*.[3] Briefly, collimated light exiting from the microscope body was split in half by a 50:50 beam splitter and sent to two Optosplit II image

splitter devices. In each Optosplit II, the light was further split by a 50:50 beam splitter, half of which passed through a sine (or cosine) filter, while the other half reached the camera unfiltered. The signal ultimately reached two identical sCMOS cameras (Prime 95b, Photometrics, operated in cooled mode), the acquisition of which is controlled by μManager software.

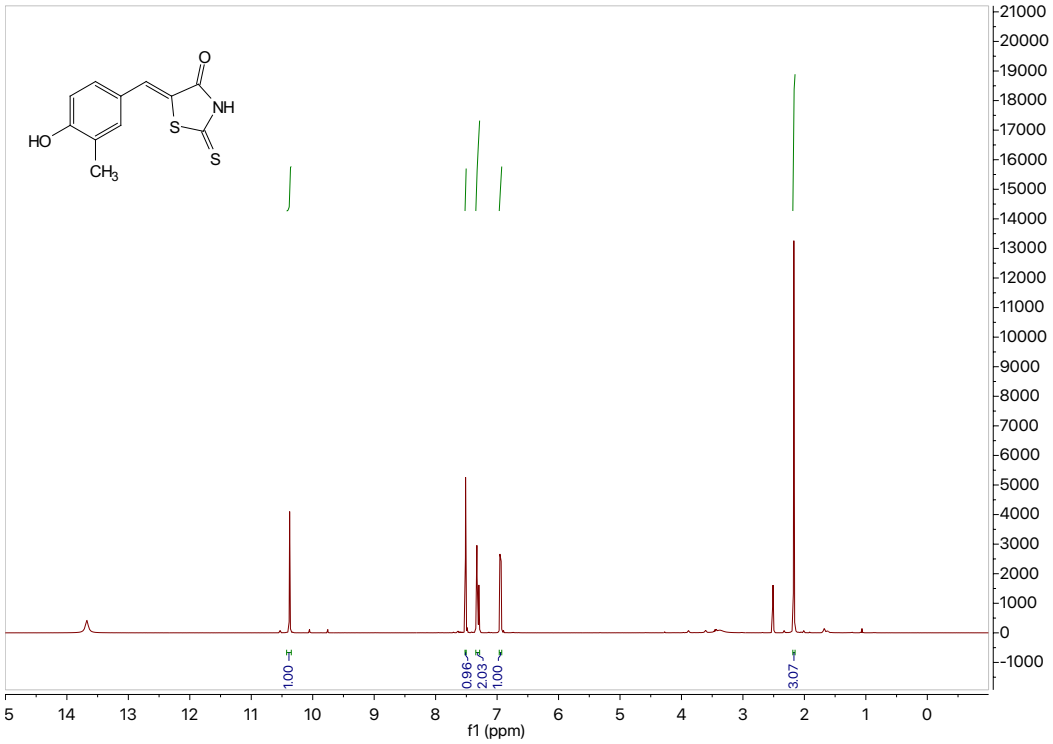
Imaging cell samples

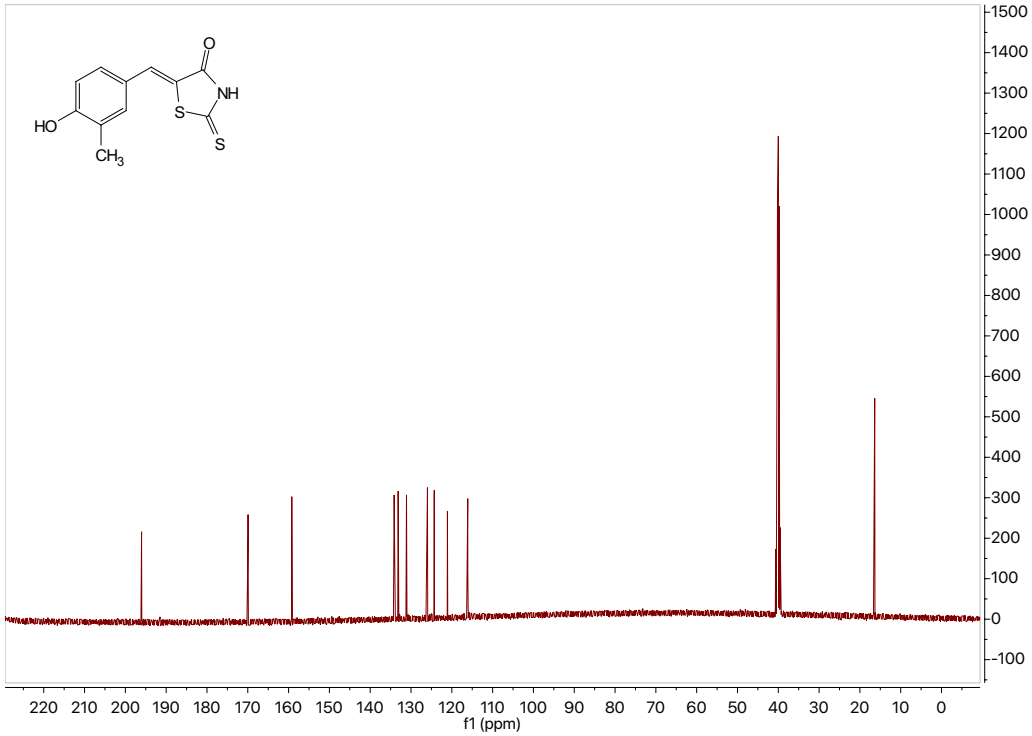
Cells were transfected with the appropriate reporter constructs and plated on an eight-well chambered coverglass slip (Ibidi). Media was aspirated and replaced with fresh media (250 μL) containing Fz (25–50 μM). Cells were then imaged 5 min post Fz addition, using the TIRF setup described above, in widefield mode (Olympus) using a ×20 air objective (Olympus UPlanSAPO ×20/0.75) with further ×2 magnification. All images were recorded with 10-s integration times and 20 frames total were collected per sample. For imaging under flow conditions, a six-channel chambered coverglass (ibidi) was plated with transfected cells 24 h before imaging. Flow channels were attached to a syringe pump (Genie plus, Kent Scientific) and run at 100 μL per minute. Media containing different ligands were separated with silicone oil, with all media containing 40 μM Fz. Images were exported and analyzed as described above and in Yao, *et al*.[3]

NMR spectra

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker spectrometers (at 400 MHz or 100 MHz), with compounds dissolved in DMSO-d_6.

HMBR





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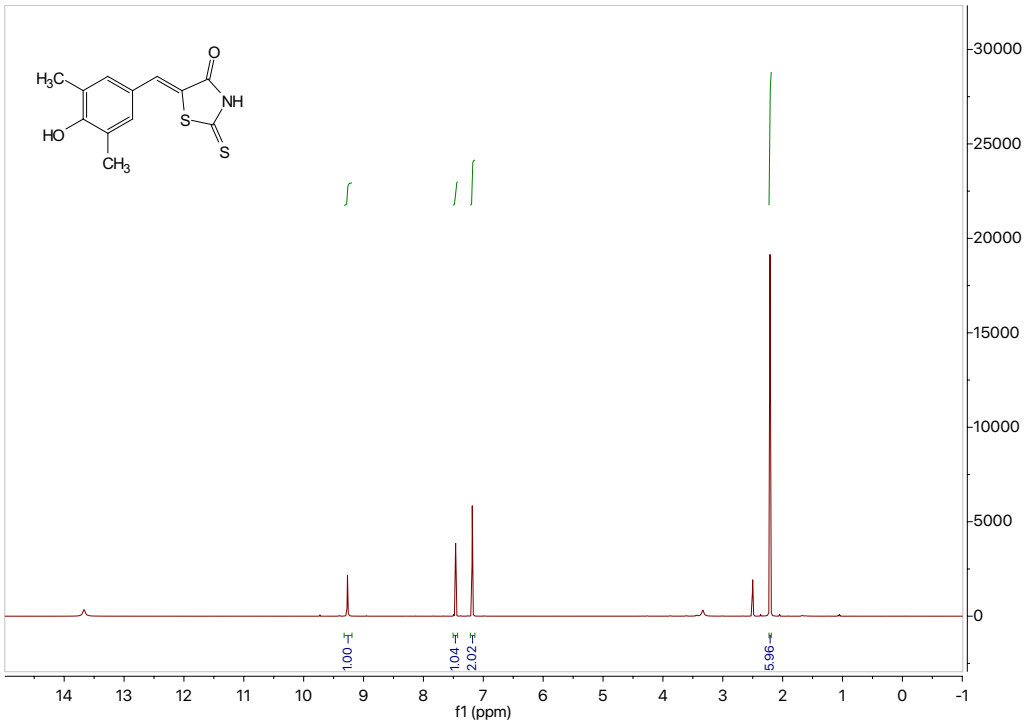
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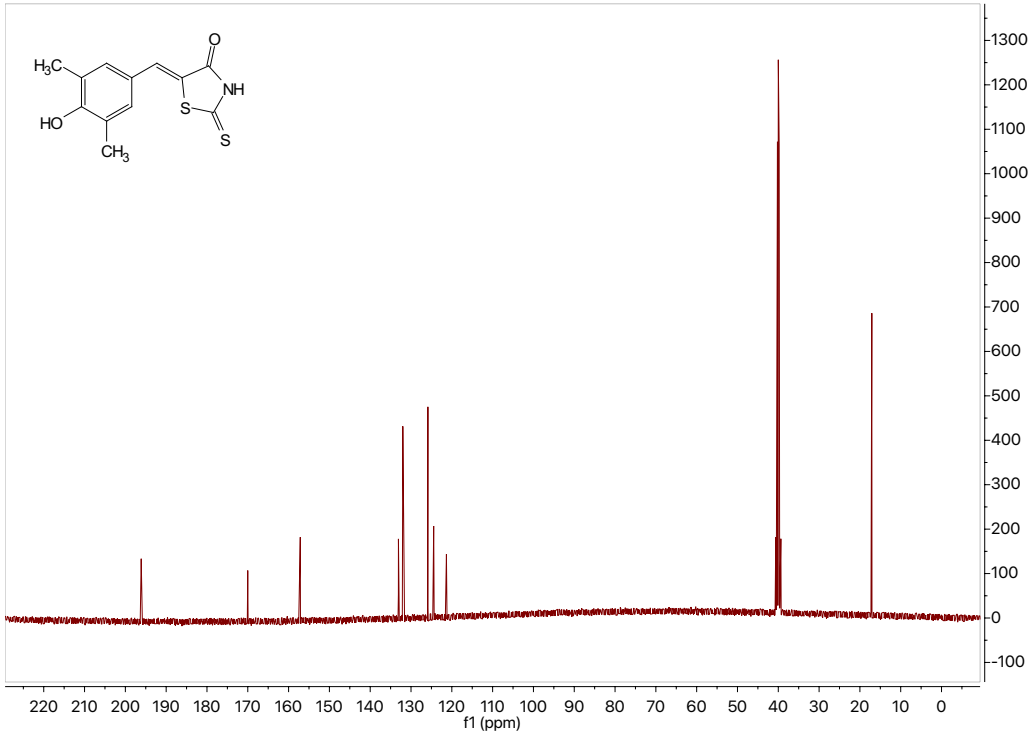
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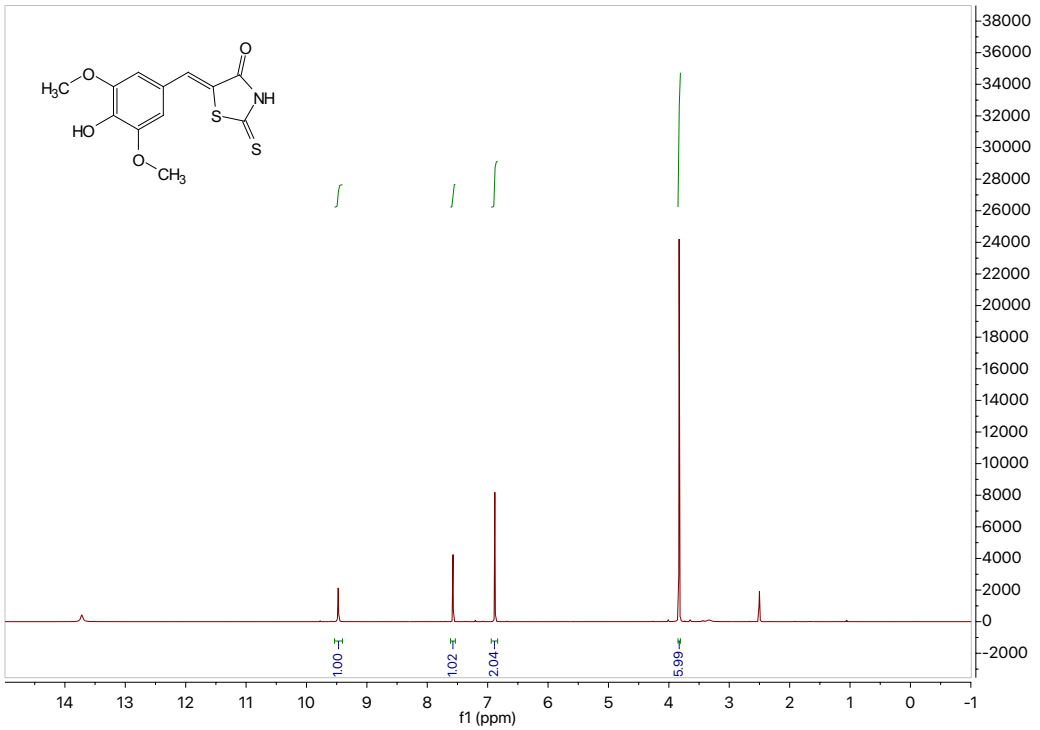
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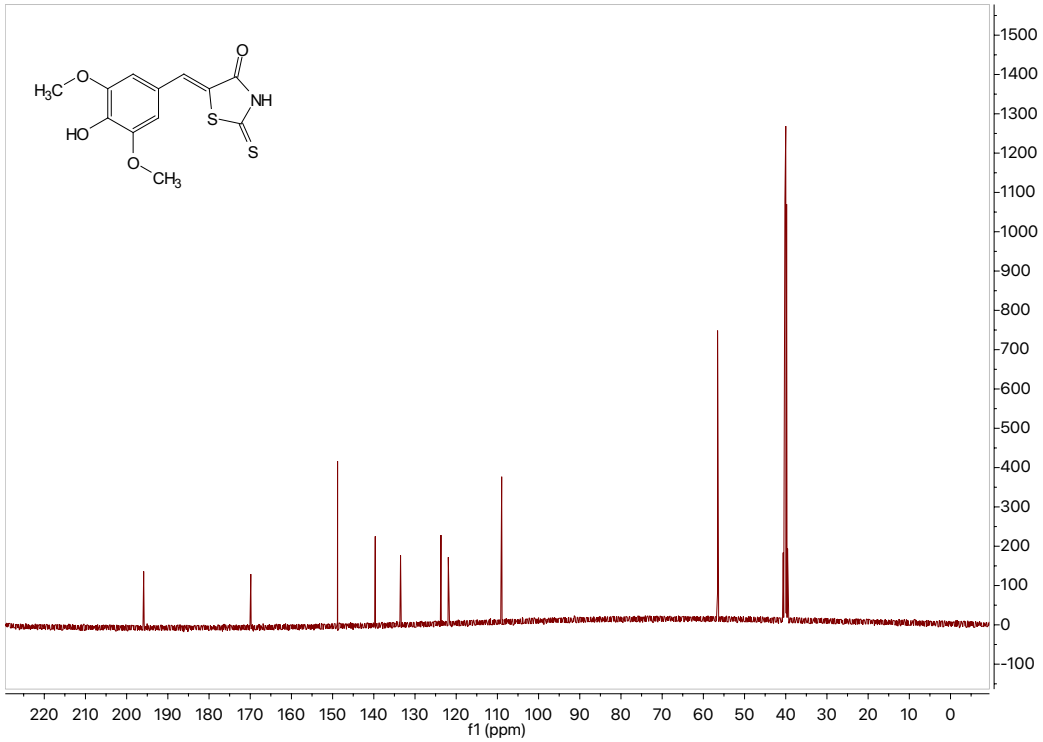
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Citations:

1 H. Benaissa, K. Ounoughi, I. Aujard, E. Fischer, R. Goïame, J. Nguyen, A. G. Tebo, C. Li, T. Le Saux, G. Bertolin, M. Tramier, L. Danglot, N. Pietrancosta, X. Morin, L. Jullien and A. Gautier, *Nat. Commun*., 2021, **12**, 6989

2 C. Li, A. Mourton, M.-A. Plamont, V. Rodrigues, I. Aujard, M. Volovitch, T. Le Saux, F. Perez, S. Vriz, L. Jullien, A. Joliot and A. Gautier, *Bioconjug. Chem.*, 2018, **29**, 1823–1828.

3 Z. Yao, C. K. Brennan, L. Scipioni, H. Chen, K. K. Ng, G. Tedeschi, K. Parag-Sharma, A. L., Amelio, E. Gratton, M. A. Digman and J. A. Prescher, *Nat. Methods*, 2022, **19**, 893–898.