Supporting Information

Engineering fluorophore recycling in a fluorogenic RNA aptamer

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Reagents and equipment

DFHBI-1T and BI fluorophores were obtained from Lucerna Technologies (New York, NY) or were synthesized as described previously.[1] Absorbance spectra were recorded with a Thermo Scientific NanoDrop 2000 spectrophotometer with cuvette capability. ChemiDoc MP imager (Bio-Rad) was used to image *in vitro* Broccoli RNA. Fluorescence was measured on FluoroMax-4 spectrofluorometer (Horiba Scientific). Fluorescence imaging experiments were performed using an Eclipse TE2000-Microscope (Nikon).

All chemicals and reagents were purchased from commercial sources without further purification, all organic solvents were used with ACS grade. All chemical reactions were performed in round-bottom flasks stirred with Teflon®-coated magnetic stir bars. The reaction progress was monitored by thin-layer chromatography (TLC) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). Analytical TLC was performed using Merck Silica gel 60 F254 pre-coated plates (0.25 mm); Illumination at 254 nm allowed the visualization of UV-active material. The ^{1}H and ^{13}C NMR spectra were recorded on a 500-MHz Bruker DMX-500 spectrometer; chemical shifts were referenced to Dimethyl sulfoxide (DMSO)-d6 (δ= 2.50 for ^{1}H NMR, δ= 39.52 for ^{13}C NMR). High-resolution mass spectra (HRMS) were recorded with Waters LCT-Premier XE.

Cells and plasmids

Human Embryonic Kidney (HEK) 293T (HEK293T, ATCC-CRL-11268) cell lines were obtained directly from the American Type Culture Collection (ATCC), and were grown in 1× DMEM (Life Technologies 11995-065) with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 g ml^{-1} of streptomycin under standard tissue culture conditions. All cells were split using TrypLE Express (Life Technologies) according to the manufacturer's instructions. Plasmids expressing Tornado Broccoli RNA were cloned according to the previous report (Addgene plasmid, 124360).[2]

Cell lines were plated for transfection using FuGENE HD (Promega, 2311), according to the manufacturer's instructions, with OptiMEM I Reduced Serum Media (ThermoFisher, 31985).

Synthesis and purification of *in vitro* Broccoli

Broccoli RNA (49b p, 5'-GAGACGGUCGGGUCCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUC-3') were synthesized by using the single-stranded DNA templates and PCR amplification with primers that included a T7 promoter DNA (19 bp, 5'-TAATACGACTCACTATAGG-3') at 5′ position to generate double-stranded DNA templates. The PCR products were then purified with PCR purification columns (Qiagen) and used as templates for *in vitro* T7 transcription reactions (Epicentre) as described previously.[3]

The synthesized Broccoli was purified from reactions using the RNA Clean & Concentrator kit (Zymo Research, R1015). Broccoli purity was determined using a precast 6% Tris-borate-EDTA (TBE)-urea gel (Life Technologies, EC68655), and run at 220-240 V in TBE buffer until completion. After staining with SYBR Gold (ThermoFisher, S11494) diluted 1:10,000 in TBE buffer, RNA bands were imaged using a ChemiDoc MP (Bio-Rad) with a preset channel (302 nm excitation and 590/110 nm emission). RNA concentration was determined using the absorbance at 260 nm by Thermo Scientific NanoDrop 2000 spectrophotometer.

Unless specified, all *in vitro* Broccoli properties were measured in 40 mM HEPES pH 7.4, 100 mM KCl, 1 mM $MgCl_2$ buffer.[4]

Quantum yield measurements

All quantum yields were determined at 25°C by comparing the integral of the corrected emission spectra for each fluorophore alone (DFHBI-1T, DFNS, BI, TBI) or Broccoli-fluorophore complex in the buffer with the corresponding integral obtained from a reference solution of DFHBI-1T or Broccoli-DFHBI-1T.[1a] Integrals at various concentrations were then plotted against the absorbance obtained at the wavelength corresponding to the excitation wavelength. The slope of this curve was compared to the slope of the curves found for reference fluorophore fluorescein. All measurements for Broccoli-fluorophore complexes were taken in the presence of excess Broccoli to avoid interference from the unbound fluorophore.

Binding affinity measurements

The dissociation constant (K_D) of the Broccoli-fluorophore complexes was determined by measuring the increase in fluorescence as a function of increasing fluorophore concentration in the presence of a fixed concentration of Broccoli aptamer as described previously.[3-5] In brief, the K_D was determined by measuring the increase in fluorescence as a function of increasing fluorophore concentration in the presence of a fixed concentration (50 nM) of *in vitro* Broccoli in the buffer at room temperature. For each concentration of fluorophore measured, a background signal for fluorophore alone was also measured and subtracted from the signal measured for Broccoli and fluorophore together. Curves were determined using nonlinear regression analysis in Origin software

and matched by least-squares fitting to a dose-response stimulation model for log(agonist) vs. normalized response -- Variable slope. The data points refer to the mean of three measurements (n = 3).

Binding and Unbinding Kinetics Measurements

The fluorescence increase was recorded with a fluorometer (Horiba) after rapid mixing of 200 nM Broccoli with different concentrations of fluorophore (DFHBI-1T, BI, DFNS, TBI) in a cuvette (400 µL). The illumination intensity was estimated to be 1−5 W/cm^2. The fluorescence intensity followed a single-exponential time course.

***In vitro* photostability measurements of Broccoli-fluorophore complexes**

To measure *in vitro* photostability measurements of Broccoli-fluorophores complex, fluorophores (0.1 µM) were mixed with excess Broccoli (1 µM) in the buffer in a cuvette (100 µl) for 10 min. The slit widths of excitation were set to maximum (29.1 nm) to make sure the entire solution volume (100 µl) was maximally illuminated. Fluorescence measurements were performed using a FluoroMax-4 spectrofluorometer (Horiba Scientific) at room temperature. The fluorescence intensities at different time points were quantified and normalized to the intensity at t = 0 sec in Origin software.

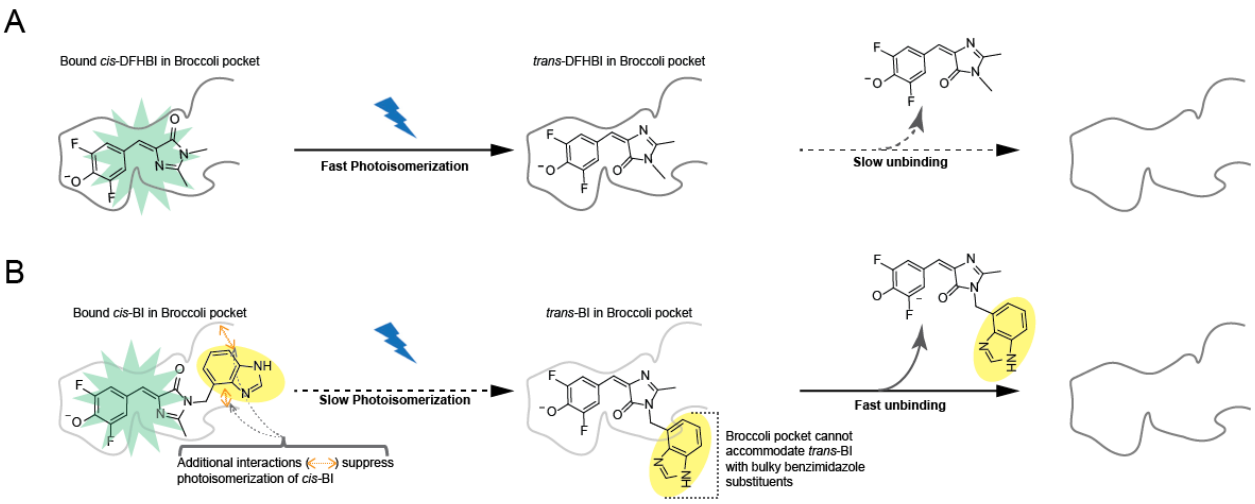
Imaging Broccoli in living mammalian cells

The first day, HEK293T cell lines were plated at a density of 5 X 10^5 cells per well of a 6-well plate in complete growth medium (DMEM + 10% FBS+100 U ml^{-1} penicillin and 100 g ml^{-1} of streptomycin). The second day, plasmids transfection. Briefly, prepared 150 µl of 0.020 µg/µl plasmid solution with OptiMEM™ I Reduced Serum Media (Thermo Fisher, 31985), added 10 µl of FuGENE® HD reagent, mixed carefully and waited for around 10 min, added the complex solutions to the HEK-293T cells, and mixed thoroughly. Meanwhile, glass-bottom 24-well plates (MatTek Corporation, P24G-1.5-13-F) were coated with poly-D-lysine (Cultrex, 3429-100-01) for overnight at 4°C. The third day, coated glass-bottom 24-well plates were illuminated under UV light for at least 10 min at room temperature and rinsed twice in 1x PBS, and additionally coated with Cultrex Mouse Laminin I (Thermo Fisher 50948048) for at least 1 h at 37° and rinsed twice in 1X PBS. Transfected cells were subcultured onto glass-bottom 24-well plates at a density of 4 X 10^4 ~8 X 10^4 cells per well. The fourth day, 2h before the imaging, the media was changed into FluoroBrite media (Thermo Fisher A1896701) containing 10 µM various fluorophores and 0.1 g/mL of Hoechst 33342 (Thermo Fisher H3570).

Live cell fluorescence images were obtained with a CoolSnap HQ2 CCD camera through a ×20 or ×40 air objective mounted on a Nikon Eclipse TE2000-E microscope and analyzed with ImageJ or NIS-Elements software. Conditions were maintained at 37 °C and 5% CO2. The filter set used for Broccoli imaging was a filter cube with excitation filter 470 ± 20 nm, dichroic mirror 495 nm (long pass), and emission filter 525 ± 25 nm. Hoechst-stained nuclei were imaged with a 350 ± 25 nm excitation filter, 400 nm dichroic mirror (long pass), and 460 ± 25 nm emission filter (all filters provided by Chroma Technology).

***In vivo* photostability measurement in mammalian cells**

Various fluorophores with different concentrations (5, 10, 20 µM) were added to the circular Broccoli RNA expressing HEK293T cells and incubated for 2 h. Images were acquired through a FITC filter using a 40× air objective at a rate of 10 frames per second and exposure time of 100 ms. The brightness was computed using ImageJ by measuring the signal in three cells' areas and subtracting background based on the average signal of control cells. Live-cell fluorescence images were taken with a CoolSnap HQ2 CCD camera using a 40× air objective mounted on a Nikon Eclipse TE2000-E microscope. Conditions were maintained at 37°C and 5% CO_2. The brightness at different time points was quantified in Origin software and normalized to the intensity at time = 0 sec. various fluorophores were added to circular Broccoli RNA-expressing HEK293T cells or control untransfected cells were imaged with a FITC filter.



Scheme S1. BI suppresses *cis-trans* photoisomerization and partly improves the recycling rate.

A) DFHBI, a Broccoli-binding fluorophore exhibits *cis-trans* photoisomerization. Because the non-fluorescent *trans*- form of DFHBI can fit in the ligand-binding pocket of Broccoli [4, 6], it exhibits slow unbinding.[1a, 7] This slow unbinding rate is a major reason for the low fluorescence of cells expressing Broccoli-DFHBI complexes.

B) BI suppresses *cis-trans* photoisomerization and accelerates the unbinding of *trans* from.[1a] BI is comprised of DFHBI and a methyl benzimidazole substituent (benzimidazole indicated in yellow ellipse). The methyl benzimidazole substituent in *cis*-BI provides additional interactions with Broccoli, which suppresses *cis-trans* photoisomerization. However, after *cis-trans* photoisomerization occurs, the *trans*- form exhibits rapid unbinding, likely due to its inability to be accommodated by the ligand-binding pocket in Broccoli.

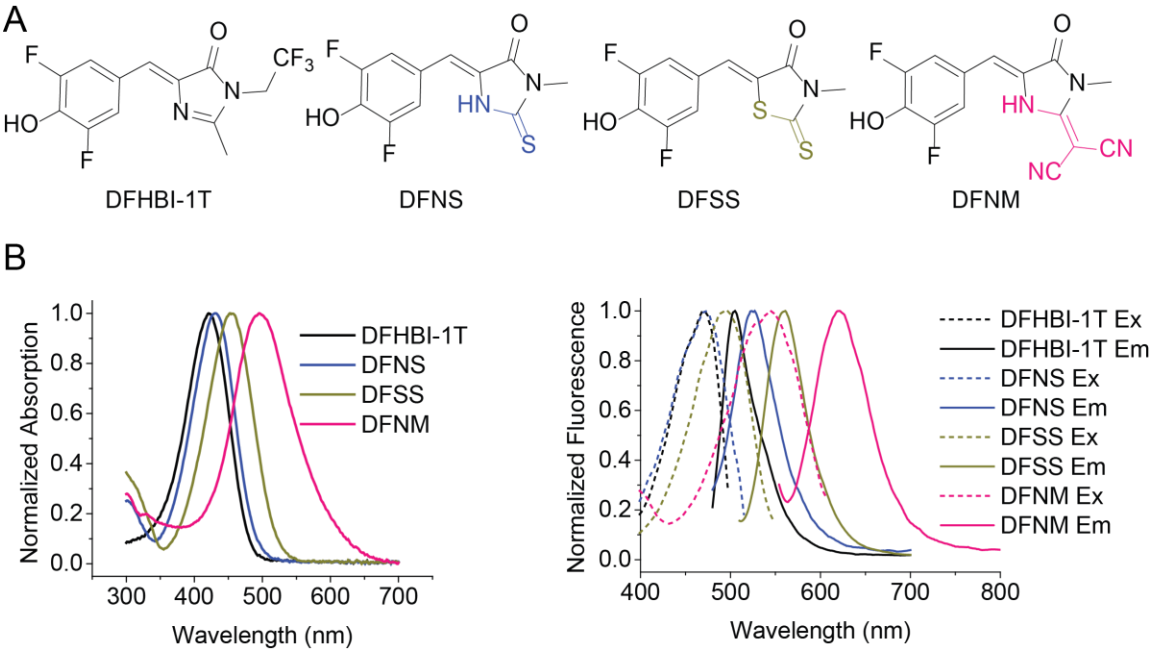


Figure S1. Atomic substitutions at C2 and N3 positions at the imidazolinone ring induce a red-shift in the fluorescence spectra relative to DFHBI-1T.

A) Chemical structure of DFHBI-1T, DFSS, DFNM and DFNS. C2 and N3 positions are shown in Fig 1A. The atomic substitutions are highlighted (dark yellow in DFSS, pink in DFNM, blue in DFNS). DFHBI-1T is shown for comparison.
B) The absorption spectra (left) and excitation/emission spectra (right) of the four fluorophores in A.

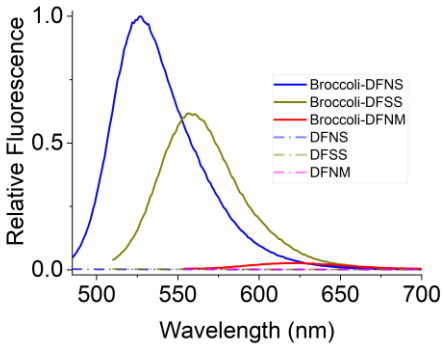


Figure S2. DFNS exhibits high fluorescence compared to DFSS and DFNM when binding to Broccoli. The emission spectra were measured using 10 μM Broccoli and 0.5 μM DFNS or DFSS or DFNM in buffer containing 40 mM HEPES pH 7.4, 100 mM KCl and 1 mM $MgCl_2$. The excitation wavelength for DFNS or Broccoli-DFNS is 470 nm; the excitation wavelength for DFSS or Broccoli-DFSS is 500 nm; the excitation wavelength for DFNM or Broccoli-DFNM is 544 nm.

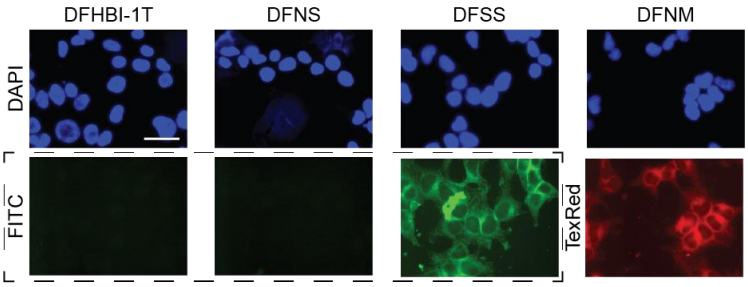


Figure S3. HEK293T cells were imaged in the presence of the indicated fluorophores (10 μM). These cells do not contain Broccoli RNA, so any fluorescence reflects nonspecific fluorescence activation by cellular components. DFSS and DFNM exhibit high background in living cells compared to DFHBI-1T and DFNS. Images were acquired with a 40× objective using a FITC filter cube for circular Broccoli RNA and a DAPI filter cube for Hoechst 33342. Exposure times: 100 ms for FITC filter; 50 ms for DAPI filter. Scale bar, 50 μm.

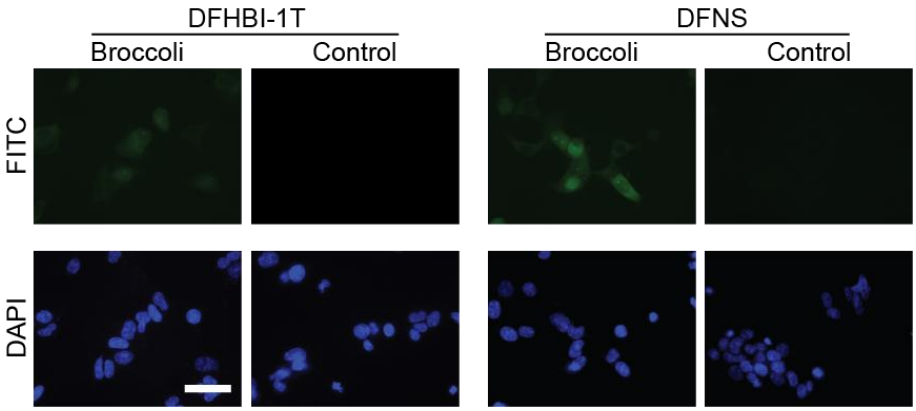


Figure S4. Comparison of cellular fluorescence of circular Broccoli-expressing HEK293T cells incubated with DFHBI-1T or DFNS. Each fluorophore was preincubated (10 μM for each compound) for 2 h with cells prior to imaging. Images were acquired with a 40× objective using a FITC filter cube for imaging circular Broccoli RNA and a DAPI filter cube for Hoechst 33342. Exposure times: 100 ms for FITC filter; 50 ms for DAPI filter. Scale bar, 50 μm.

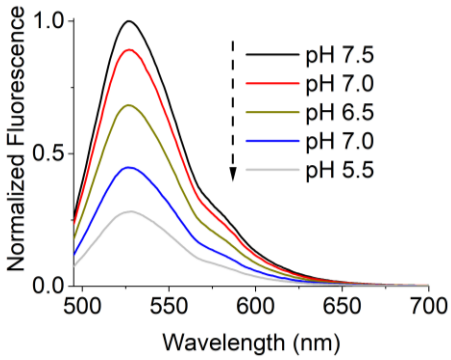


Figure S5. The fluorescence of Broccoli-TBI complex decreases with lower pH. The emission spectra were measured using 10 μM Broccoli and 1 μM TBI in buffer containing 40 mM HEPES at the indicated pH, 100 mM KCl and 1 mM $MgCl_2$. The excitation wavelength for Broccoli-TBI is 485 nm. Minimal effects are seen at pH 7.0, but prominent reduction in fluorescence is seen at lower pH. This may reflect poor folding of Broccoli at lower pH, or protonation of TBI, which would result in the lower fluorescence phenol-form of TBI.[3, 8]

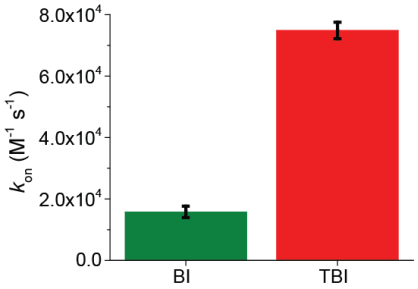


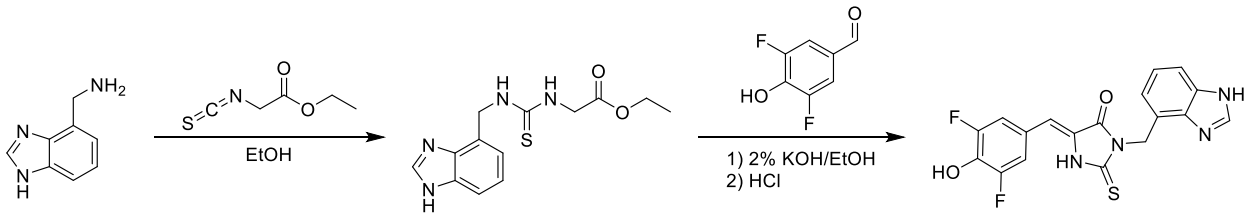
Figure S6. TBI exhibits higher k_{on} compared to BI fluorophore. k_{on} was measured as described previously.[1a, 7a] The resulting constants were k_{on} = 15,800 M^{-1} s^{-1} for BI and k_{on} = 74,900 M^{-1} s^{-1} for TBI. These results show that TBI binds Broccoli more rapidly compared to BI.

Table S1. DNA sequences used for in vitro transcription.

Name	Sequences (5'-3')
T7 promoter	TAATACGACTCACTATAGG
Broccoli[4]	GAGACGGTCGGGTCCAGATATTCGTATCTGTCGAGTAGAGTGTGGGCTC

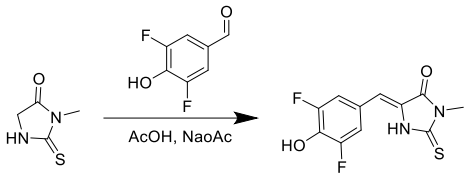
Fluorophore Synthesis

Synthesis of (Z)-3-((1H-benzo[d]imidazol-4-yl)methyl)-5-(3,5-difluoro-4-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (TBI)



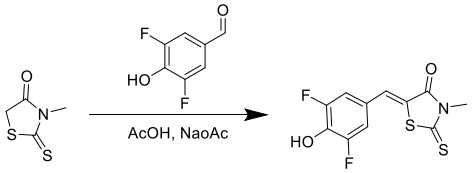
TBI synthetic route was followed by the reported reference.[9] Ethyl isothiocyanatoacetate (0.61 mmol, 88 mg) and (1H-Benzo[d]imidazol-4-yl)methanamine hydrochloride (0.61 mmol, 111.7 mg) were mixed in 3ml pure EtOH and stirred overnight at room temperature. The precipitate was filtered, washed with cold Et_2O, and dried to get the thioureas product ethyl (((1H-benzo[d]imidazol-4-yl)methyl)carbamothioyl)glycinate (166.8 mg). Then, the thioureas product (0.44 mmol, 130 mg) was added in 12 mL of 2% KOH in ethanol with 3,5-difluoro-4-hydroxybenzaldehyde (0.44 mmol, 70 mg). The reaction mixture was stirred at room temperature overnight, and then 10% HCl was slowly added. The precipitate was collected by filtration and washed with cold ethanol, cold water, hexane and dried to afford TBI (38.9 mg, yield 23%).^{1}H NMR (500 MHz, DMSO-d_6) δ 12.53 (s, 2H), 10.90 (s, 1H), 8.25 (s, 1H), 7.69 – 7.57 (m, 2H), 7.45 (s, 1H), 7.11 (t, J = 7.8 Hz, 1H), 6.78 (s, 1H), 6.59 (s, 1H), 5.38 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 178.68, 174.48, 164.15, 161.69, 153.07, 153.01, 151.15, 151.09, 141.79, 135.32, 122.65, 121.78, 114.03, 113.98, 113.90, 113.85, 111.51, 28.92. High-resolution MS: m/z calculated for C18H12F2N4O2S [M+H]+ 387.0727, found: 387.0708.

Synthesis of (Z)-5-(3,5-difluoro-4-hydroxybenzylidene)-3-methyl-2-thioxoimidazolidin-4-one (DFNS)



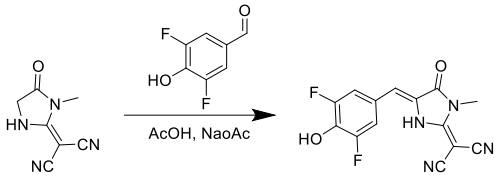
3-methyl-2-thioxoimidazolidin-4-one (87 mg,0.67mmol), 3,5-difluoro-4-hydroxybenzaldehyde (91 mg,0.67mmol), and anhydrous sodium acetate (82,0.67mmol) were stirred in acetic acid at 120°C for 3 hours. After allowing the reaction to cool to room temperature, acetic acid was added while stirring and the reaction was left stirring overnight. The resulting crystalline solid was then washed with a small amount of acetic acid, hot water, hexanes and dried to afford DFNS (121.5 mg. yield 67%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.40 (s, 1H), 10.86 (s, 1H), 7.60 (dd, *J* = 8.2, 1.7 Hz, 2H), 6.56 (s, 1H), 3.21 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 178.96, 164.06, 153.02, 151.10, 135.19, 125.60, 122.74, 114.01, 113.83, 111.15, 27.24. High-resolution MS: m/z calculated for C11H8F2N2O2S [M-H]$^-$ 269.0196, found: 269.0197.

Synthesis of (Z)-5-(3,5-difluoro-4-hydroxybenzylidene)-3-methyl-2-thioxothiazolidin-4-one (DFSS)



3-methyl-2-thioxothiazolidin-4-one (257 mg,1.75 mmol), 3,5-difluoro-4-hydroxybenzaldehyde (238 mg,1.75 mmol), and anhydrous sodium acetate (143.5 mg, 1.75 mmol) were stirred in acetic acid at 130°C for 2 hours. After allowing the reaction to cool to room temperature, acetic acid was added while stirring and the reaction was left stirring overnight. The resulting crystalline solid was then washed with a small amount of acetic acid, hot water, hexanes and dried to afford DFSS (278 mg, yield 55%).^{1}H NMR (500 MHz, DMSO-d_6) δ 11.33 (s, 1H), 7.73 (s, 1H), 7.37 (d, *J* = 7.8 Hz, 2H), 3.39 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 193.01, 166.91, 153.13, 151.20, 136.77, 131.06, 123.30, 121.77, 114.40, 114.21, 31.19. High-resolution MS: m/z calculated for C11H7F2NO2S2 [M-H]$^-$ 285.9808, found: 285.9805.

Synthesis of (Z)-2-(4-(3,5-difluoro-4-hydroxybenzylidene)-1-methyl-5-oxoimidazolidin-2-ylidene)malononitrile (DFNM)



2-(1-methyl-5-oxoimidazolidin-2-ylidene)malononitrile (76 mg,0.47 mmol), 3,5-difluoro-4-hydroxybenzaldehyde (57 mg,0.36 mmol), and anhydrous sodium acetate (35 mg) were stirred in acetic acid at 130°C for 2 hours. After allowing the reaction to cool to room temperature, acetic acid was added while stirring and the reaction was left stirring overnight. The resulting crystalline solid was then washed with a small amount of acetic acid, hot water, hexanes and

dried to afford DFSS (66 mg, yield 61%).^{1}H NMR (500 MHz, DMSO-d_6) δ 10.64 (s, 1H), 7.40 (d, J = 8.7 Hz, 2H), 6.45 (s, 1H), 2.95 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.11, 155.27, 153.05, 151.13, 126.18, 123.38, 113.09, 107.76, 43.73, 24.26. High-resolution MS: m/z calculated for C14H8F2N4O2 $[M-H]^-$ 301.0543, found: 301.0541.

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