SUPPORTING INFORMATION

Plug-and-play fluorophores extend the spectral properties of Spinach

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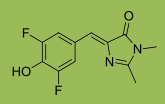
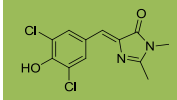
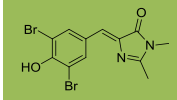
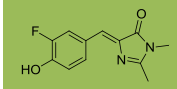
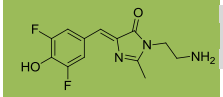
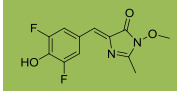
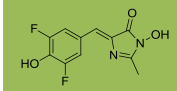
I- Supplemental Table and Figure

Table S1. Photophysical and binding properties of Fluorophores and Fluorophore-Spinach2 complexes

	Fluorophore	Excitation maximum (nm)	Emission maximum (nm)	Extinction coefficient[a] ($M^{-1}cm^{-1}$)	Fluorescence quantum yield	K_D (nM)	Bright-ness[b]
	DFHBI	423	489	30,100	0.0007	-	0.13
	Spinach2-DFHBI	447	501	22,000	0.72	530	100
	DClHBI	424	490	19,000	0.0015	-	0.18
	Spinach2-DClHBI	447	500	18,000	0.42	1200	48
	DBrHBI	431	500	34,800	0.0012	-	0.26
	Spinach2-DBrHBI	446	499	27,000	0.41	950	70
	FHBI	430	498	27,900	0.0009	-	0.16
	Spinach2-FHBI	464	501	24,000	0.56	660	85
	DFHBI-AE	429	496	24,700	0.00086	-	0.13
	Spinach2-DFHBI-AE	477	510	18,000	0.55	2200	63
	DFHBI-1MO	430	501	22,200	0.0011	-	0.15
	Spinach2-DFHBI-1MO	471	503	21,000	0.33	2000	44
	DFHBI-1HO	N.D.[c]	N.D.	11,200	N.D.	-	N.D.
	Spinach2-DFHBI-1HO	454	504	3,000	0.02	8100	0.38

[a]Extinction coefficients were all measured at pH7.4 using the conditions described in Figure 1. [b]Brightness (extinction coefficient × quantum yield) is reported relative to Spinach2-DFHBI. [c]N.D = not detectable

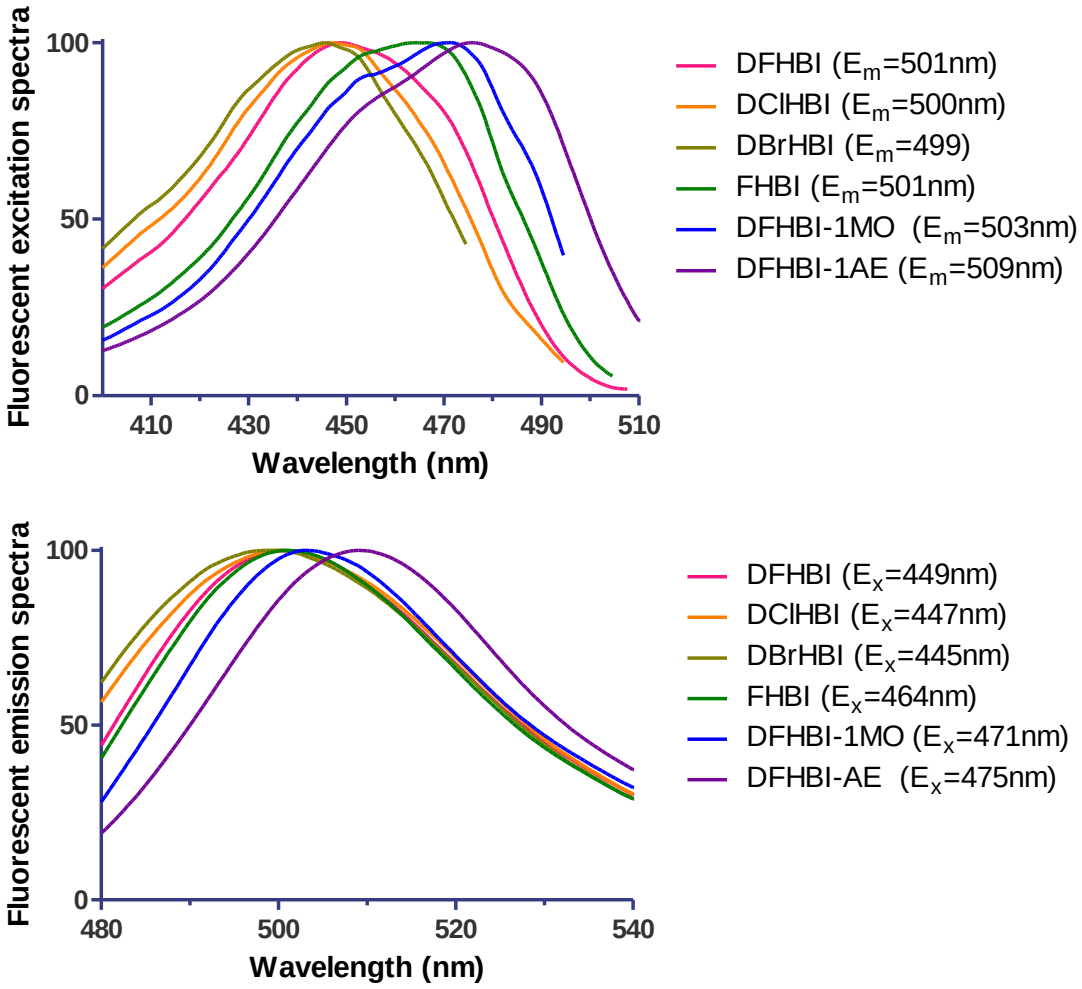


Figure S1. Different fluorophores tune the fluorescence spectral properties of Spinach2. In these experiments 5 µM Spinach2 RNA was incubated with 1 µM of the indicated fluorophore. The excitation spectra were obtained using the indicated emission wavelength (E_m). The emission spectra were obtained using the indicated excitation wavelength (E_x). The spectra were normalized to 100% for comparison.

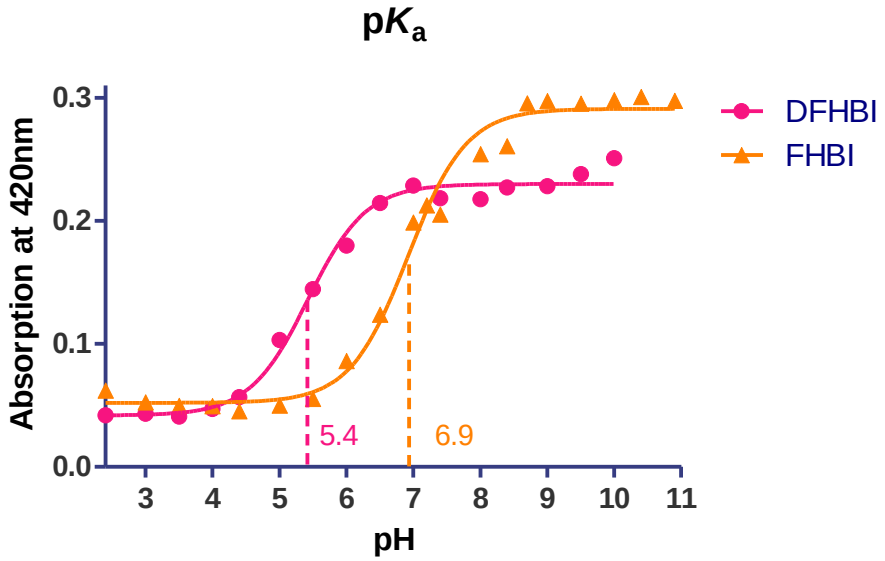


Figure S2. pH-titration of fluorophores DFHBI and FHBI. The purpose of this experiment was to establish the pK_a of FHBI. pK_as of compounds **2** (DFHBI) and **8** (FHBI) were measured by monitoring the absorbance of the phenolic peak at 420 nm. Titration of ~ 20 μM fluorophore was performed in different pH buffers as described previously.[1] The pK_a was established by first determining the baseline absorbance and the maximal absorbance for each fluorophore. The pK_a was determined as the pH at which the phenolate absorbance curve gives a response half way between the baseline and maximal absorbance for each fluorophore (indicated with dotted line).

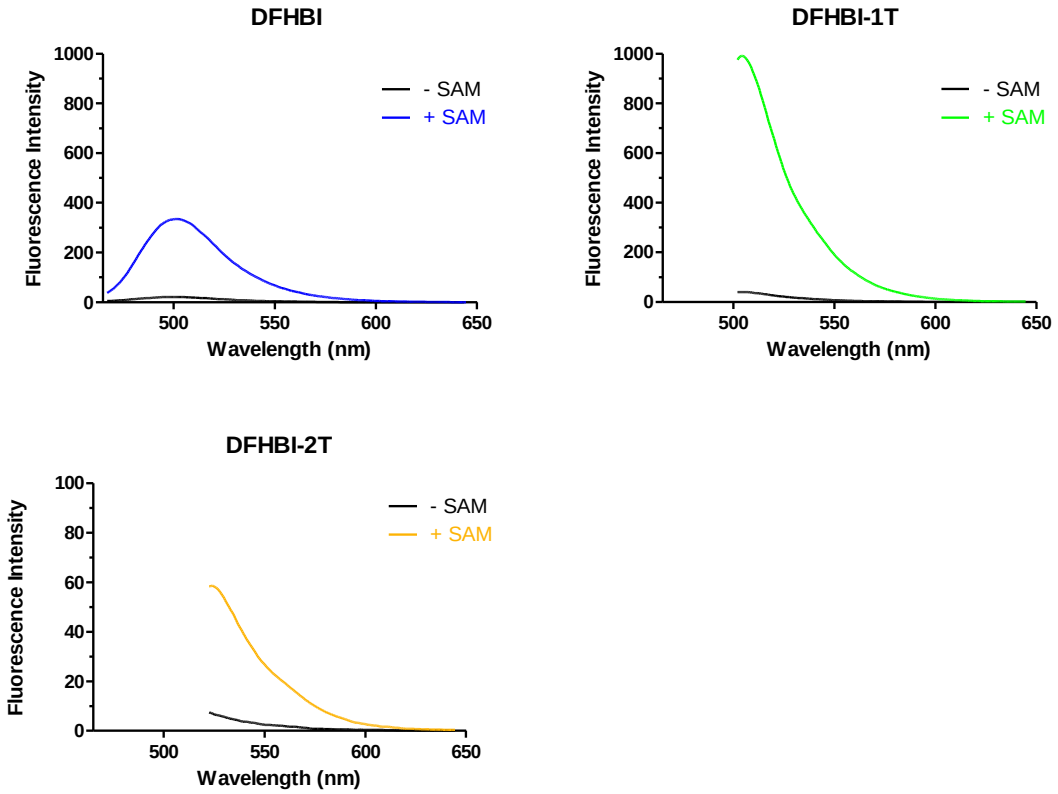


Figure S3: Different fluorophores allow the detection of S-adenosylmethionine (SAM) using different excitation and emission wavelengths. Shown is the emission spectra of fluorophore-Spinach2-SAM sensor complexes (2 µM RNA, 10 µM fluorophore) plus and minus 1 mM SAM. Spectra were collected in the presence of excess fluorophore at pH 7.4 (37°C, 30 min) for RNAs binding to DFHBI (blue), DFHBI-1T (green), and DFHBI-2T (yellow). Fluorescence emission was recorded using the following instrumental parameters: Ex, DFHBI = 460 nm, Ex, DFHBI-1T = 482 nm, Ex, DFHBI-2T = 500 nm; slit widths, 10 nm.

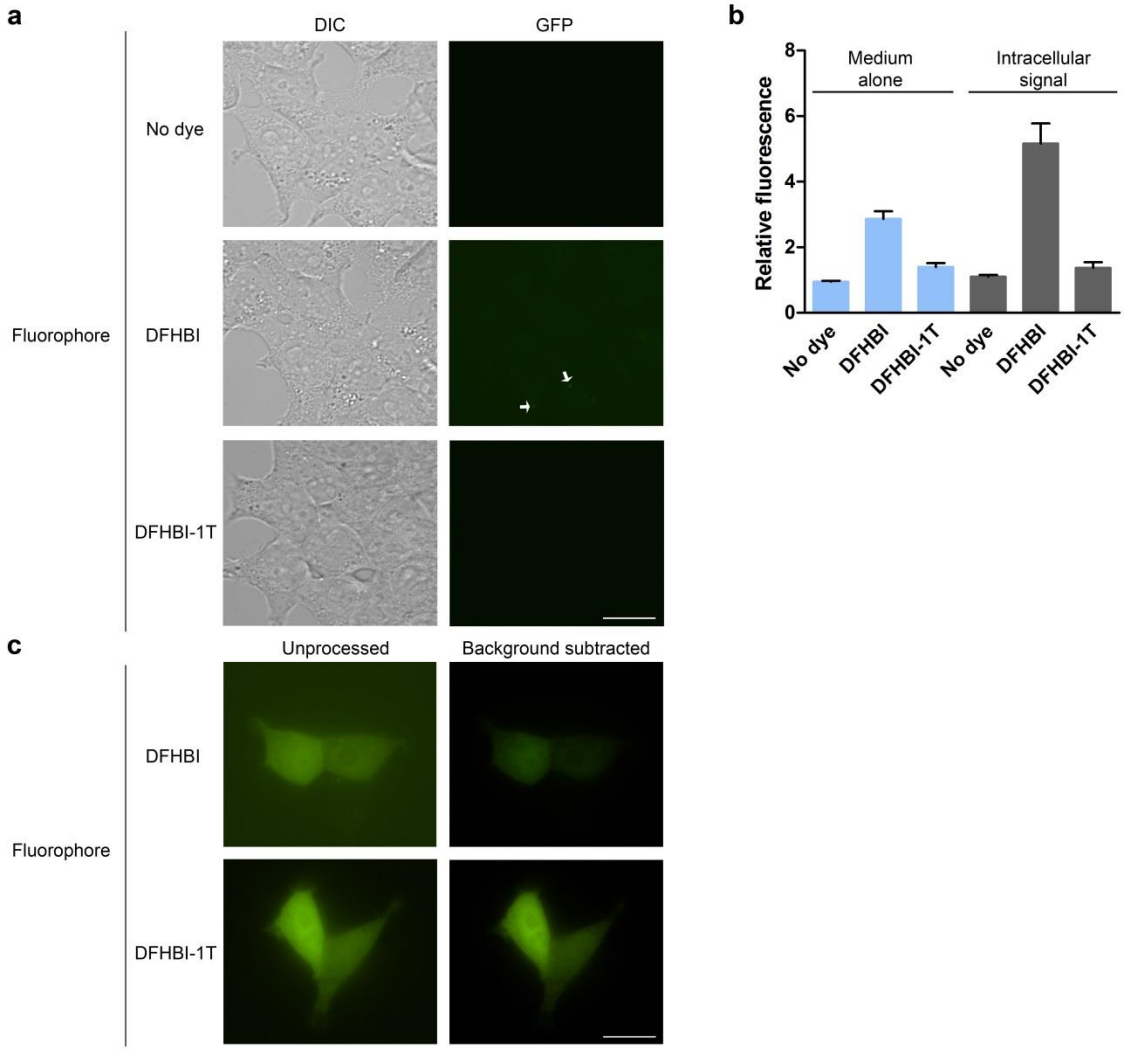


Figure S4. Comparison of background fluorescence values in the presence of different fluorophores. (a) Untransfected HEK-293T cells were incubated with imaging media containing either no fluorophore, 20 μM DFHBI, or 20 μM DFHBI-1T for 30 min. Shown are DIC (left column) and FITC fluorescence images (right column) after 1 sec exposures. Although minimal fluorescence is seen with DFHBI, there is a faint green haze that reflects low level DFHBI autofluorescence in the media. Additionally, there are faint debris-like structures that can occasionally be seen (white arrows), which appear to reflect nonspecific fluorescence activation of DFHBI. In cells cultured with 20 μM DFHBI-1T, the faint green haze is less prominent and there the interaction of DFHBI-1T with intracellular structures is not readily detectable. (b) Quantification of background fluorescence signal in the presence of different fluorophores. Signal was quantified from

images acquired after 1 sec exposures of samples containing imaging medium alone (baseline) or imaging medium supplemented with 20 μM DFHBI or DFHBI-1T (light blue bars). Signal was also quantified in untransfected cells to determine background cellular fluorescence. For these quantifications, fluorescence was quantified after 1 sec exposures from untransfected cells incubated in imaging medium alone or imaging medium supplemented with 20 μM DFHBI or DFHBI-1T (gray bars). Fluorescence values were normalized to the baseline signal obtained from imaging media with no fluorophore. One second exposure times were required in order to obtain readily quantifiable background fluorescence measurements. DFHBI-1T showed markedly reduced background signals in both imaging media and in cells relative to DFHBI. Shown are s.e.m. values for three independent image fields per sample. (c) HEK-293T cells transfected to express 5S-Spinach2 were imaged in the presence of 20 μM DFHBI or DFHBI-1T. Shown are fluorescence images acquired for 100 msec. Shown are images before (left column) and after (right column) background subtraction. As can be seen for cells cultured with DFHBI, there is a green haze, and background subtraction of this haze improves the detection of 5S-Spinach2. However, for cells cultured with DFHBI-1T, there is lower background fluorescence, so background subtraction does not produce an image that is as noticeably enhanced.

II- Experimental Section

A. General

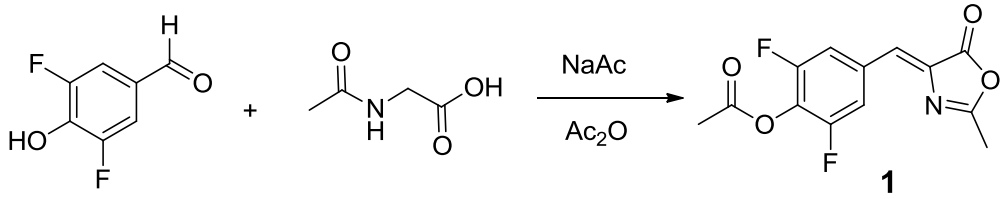
The ^{1}H and ^{13}C NMR spectra were recorded on a 500-MHz Bruker DMX-500 spectrometer; chemical shifts were referenced to an internal tetramethylsilane standard (δ = 0.0 for ^{1}H NMR) or the residual solvent peak (for ^{13}C NMR). LC/MS (MS: ESI+) was performed on a Waters Acquity ultra-performance liquid chromatography (UPLC) system connected to a Waters Micromass SQ electrospray ionization (ESI) spectrometer. UPLC was performed at a flow rate of 0.5 ml/min (monitored with a PDA from 210-500 nm) using a Waters C18 column. High Resolution Mass Spectra (HR MS) were recorded with Waters LCT-Premier XE. UV-VIS spectra were recorded with a Thermo Scientific NanoDrop 2000 spectrophotometer with 1 cm light pass cuvette capability. Fluorescence

excitation and emission spectra were measured with a Perkin Elmer LS-55 fluorescence spectrometer.

All chemicals and reagents were purchased from commercial sources without further purification. Thin layer chromatography was performed on EMD Silica Gel 60 F_{254}. Flash chromatography was conducted with 200-400 mesh silica gel 60 (EMD) and with ACS or HPLC grade solvents.

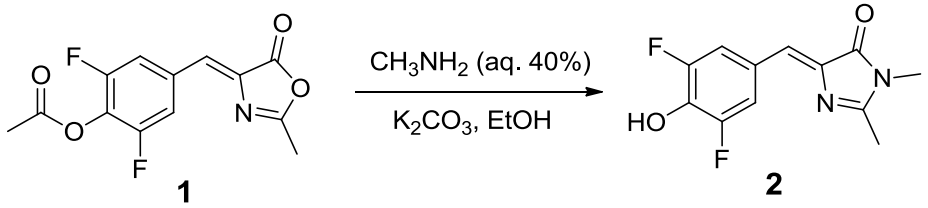
B. Fluorophore Synthesis

Synthesis of (*Z*)-2,6-difluoro-4-((2-methyl-5-oxooxazol-4(5*H*)-ylidene)methyl)phenyl acetate (**1**)[1]:



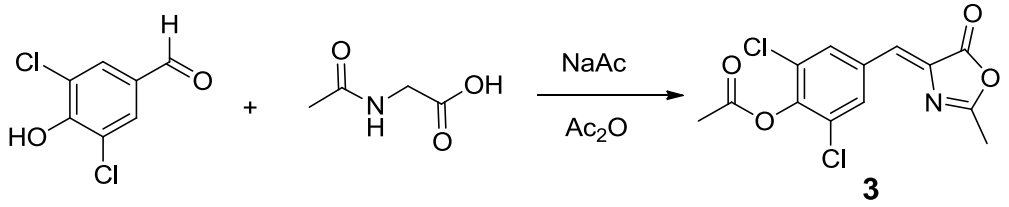
Compound **1-8, 11,** and **12** was synthesized as described previously[1]. N-Acetylglycine (2.22 g, 18.99 mmol), anhydrous sodium acetate (1.56 g, 18.99 mmol), 4-hydroxy-3,5-difluorobenzaldehyde (3.00 g, 18.99 mmol), and acetic anhydride (7.2 ml) were stirred at 100 ℃ for 1 h. After allowing the reaction to cool to room temperature, cold ethanol (30 ml) was added while stirring and the reaction was left stirring overnight at 4 ℃. The resulting crystalline solid was then washed with a small amount of cold ethanol, hot water, hexanes and dried to afford 3.40 g (yield 64%) of **1** as a pale yellow solid: ^{1}H NMR (500 MHz, CDCl3) δ 7.77 (d, J = 8.45Hz, 2H), 6.96 (s, 1H), 2.43 (s, 3H), 2.40 (s, 3H); LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.48 min; purity, 95%; 282.35 $[M+H]^+$. The NMR and MS spectrum are the same as previously described[1].

Synthesis of (*Z*)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5(4*H*)-one (**2**, DFHBI):



Compound **1** (728 mg, 2.59 mmol) was refluxed with 10 ml of ethanol, 0.73 ml of 40% aqueous methylamine, and 510 mg of potassium carbonate for 4 h. The reaction mixture was removed from heat and upon cooling formed an orange precipitate. The precipitate containing the product was filtered and washed briefly with cold ethanol. The precipitate was then redissolved in a 1:1 mixture of ethyl acetate and 500 mM sodium acetate pH 3.0. The organic layer was separated, dried with anhydrous sodium sulfate and solvent was removed under reduced pressure to afford 392 mg (yield 60%) of DFHBI as a bright yellow solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.94(s, 1H), 7.97 (d, J = 9.5, 2H), 6.90 (s, 1H), 3.09 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 15.44 (CH_3), 26.29 (CH_3), 115.16 (dd, J = 16.36Hz, 6.27Hz, CH×2), 122.80 (CH), 124.70 (t, J = 9.28Hz), 135.72 (t, J = 16.30Hz), 138.43, 151.84 (dd, J = 241.33Hz, 7.4Hz), 164.51, 169.63; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 1.79 min; purity, 95%; 253.18 $[M+H]^+$.

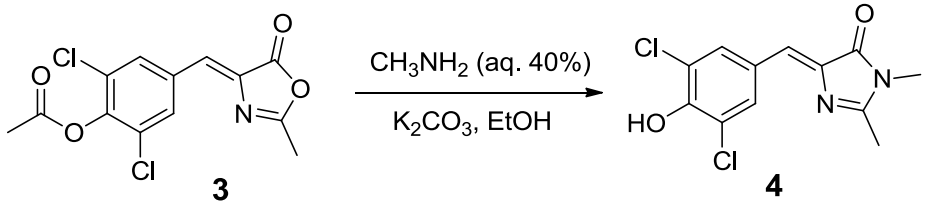
Synthesis of (*Z*)-2,6-dichloro-4-((2-methyl-5-oxooxazol-4(5*H*)-ylidene)methyl)phenyl acetate (**3**):



N-Acetylglycine (674 mg, 5.76 mmol), anhydrous sodium acetate (473 mg, 5.76 mmol), 4-hydroxy-3,5-dichlorobenzaldehyde (1.00 g, 5.24 mmol), and acetic anhydride (3.5 ml) were stirred at 100 ℃ for 1 h. After allowing the reaction to cool to room temperature, cold ethanol (10 ml) was added while stirring and the reaction was left stirring overnight at 4 ℃. The resulting crystalline solid was then washed with a small amount of cold ethanol, hot water, hexanes and dried to afford 1.23 g (yield 75%) of **3** as a pale yellow

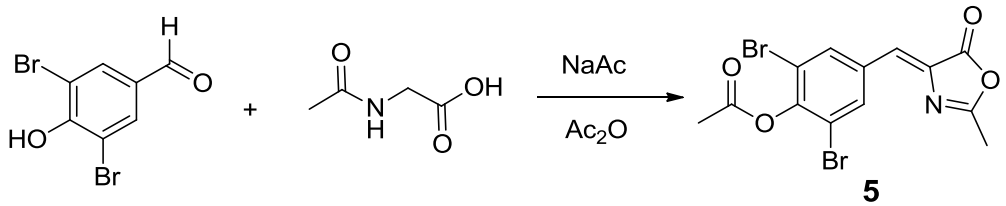
solid: ^{1}H NMR (500 MHz, CDCl3) δ 8.11 (s, 2H), 6.94 (s, 1H), 2.43 (s, 3H), 2.42 (s, 3H) ; ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 15.52 (CH_3), 19.88 (CH_3), 125.28, 128.27 (CH×2), 131.40 (CH×2), 133.23, 134.79, 144.32, 166.74, 167.21, 168.57.

Synthesis of (*Z*)-4-(3,5-dichloro-4-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5(4*H*)-one (**4**, DClHBI):



Compound **3** (314 mg, 1.0 mmol) was refluxed with 5 ml of ethanol, 0.4 ml of 40% aqueous methylamine, and 208 mg of potassium carbonate for 4 h. The reaction mixture was removed from heat and cooled to room temperature, diluted with 50ml of water, acidified with concentrated HCl to pH 3. The result solid were filtered and purified by column chromatography (CH_2Cl_2:EtOH = 10:1) to afford 174 mg (yield 61%) of **4** as yellow solid: ^{1}H NMR (500 MHz, CD_3OD) δ 8.11 (s, 2H), 6.87 (s, 1H), 3.18 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 15.43 (CH_3), 26.88 (CH_3), 123.59, 125.29 (CH), 128.32, 133.16 (CH×2), 139.54, 152.61, 165.70, 172.09; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.31 min; purity, 95%; 285.21 $[M+H]^+$.

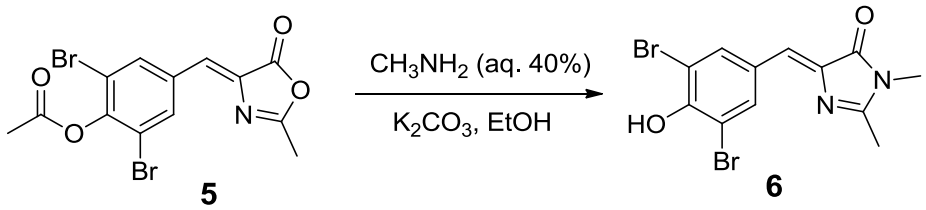
Synthesis of (*Z*)-2,6-dibromo-4-((2-methyl-5-oxooxazol-4(5*H*)-ylidene)methyl)phenyl acetate (**5**):



N-Acetylglycine (420 mg, 3.58 mmol), anhydrous sodium acetate (290 mg, 3.58 mmol), 4-hydroxy-3,5-dibromobenzaldehyde (1.00 g, 3.58 mmol), and acetic anhydride (2.5 ml) were stirred at 100 ℃ for 1 h. After allowing the reaction to cool to room temperature,

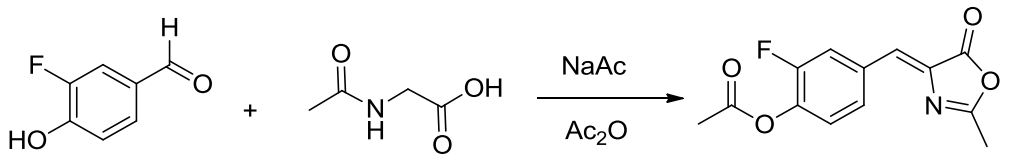
cold ethanol (5 ml) was added while stirring and the reaction was left stirring overnight at 4 ℃. The resulting crystalline solid was then washed with a small amount of cold ethanol, hot water, hexanes and dried to afford 800 mg (yield 55%) of **5** as a yellow solid: ^{1}H NMR (500 MHz, CDCl3) δ 8.30 (s, 2H), 6.94 (s, 1H), 2.44 (s, 3H), 2.42 (s, 3H) ; ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 15.53 (CH_3), 20.18 (CH_3), 117.55, 125.17 (CH×2), 134.11, 134.66, 134.99 (CH×2), 146.67, 166.73, 167.09, 168.49; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 3.0 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.62 min; purity, 95%; 404.14 $[M+H]^+$.

Synthesis of (*Z*)-4-(3,5-dibromo-4-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5(4*H*)-one (**6**, DBrHBI):



Compound **5** (200 mg, 0.5 mmol) was refluxed with 4 ml of ethanol, 0.2 ml of 40% aqueous methylamine, and 104 mg of potassium carbonate for 4 h. The reaction mixture was removed from heat and cooled to room temperature. The solvent was evaporated and the product was redissolved in a 1:1 mixture of ethyl acetate and 500 mM sodium acetate pH 3.0. The organic layer was separated, dried with anhydrous sodium sulfate and solvent was removed under reduced pressure to afford 75 mg (yield 40%) of DBrHBI as a brown solid: ^{1}H NMR (500 MHz, CD_3OD) δ 8.31 (s, 2H), 6.87 (s, 1H), 3.18 (s, 3H), 2.38 (s, 3H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 15.40 (CH_3), 26.84 (CH_3), 112.20, 124.92 (CH), 129.71, 136.95 (CH×2), 139.50, 154.19, 165.68, 172.03; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.01 min; purity, 95%; 375.03 $[M+H]^+$.

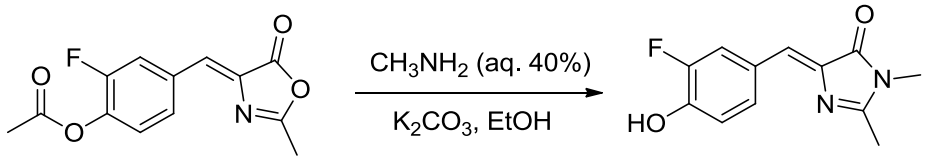
Synthesis of (*Z*)-2-fluoro-4-((2-methyl-5-oxooxazol-4(5*H*)-ylidene)methyl)phenyl acetate (**7**):



7

N-Acetylglycine (919 mg, 7.86 mmol), anhydrous sodium acetate (644 mg, 7.86 mmol), 3-fluoro-4-hydroxybenzaldehyde (3.00 g, 18.99 mmol), and acetic anhydride (3 ml) were stirred at 100 ℃ for 1 h. After allowing the reaction to cool to room temperature, cold ethanol (10 ml) was added while stirring and the reaction was left stirring overnight at 4 ℃. The resulting crystalline solid was then washed with a small amount of cold ethanol, hot water, hexanes and dried to afford 1.07 g (yield 57%) of **7** as a yellow solid: ^{1}H NMR (500 MHz, CDCl3) δ 8.15 (dd, J = 13.05Hz, 1.9Hz, 1H), 7.70 (d, J =8.35 Hz, 1H), 7.20 (t, J =8.05 Hz, 1H), 7.05 (s, 1H), 2.42 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 15.43 (CH_3), 20.23 (CH_3), 118.95 (d, J = 20.12Hz, CH), 124.69, 127.27, 128.97 (d, J = 3.13Hz, CH), 132.57 (d, J = 7.7Hz), 133.60, 139.29 (d, J = 13.4Hz), 153.29 (d, J = 247.08Hz), 167.06, 167.67, 168.02.

Synthesis of (*Z*)-4-(3-fluoro-4-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5(4*H*)-one (**8**, FHBI):

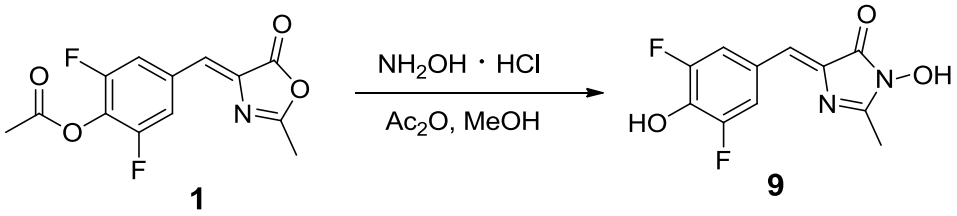


7 **8**

Compound **7** (263 mg, 1.0 mmol) was refluxed with 2 ml of ethanol, 0.4 ml of 40% aqueous methylamine, and 208 mg of potassium carbonate for 4 h. The reaction mixture was removed from heat and cooled to room temperature, diluted with 25ml of water, acidified with concentrated HCl to pH 3. The result solid were filtered and recrystallized by ethanol to afford 97 mg (yield 41%) of **8** as yellow solid: ^{1}H NMR (500 MHz, CD_3OD) δ 10.57(s, 1H), 8.20 (dd, J = 13.05Hz, 1.4Hz, 1H), 7.76 (d, J =8.4 Hz, 1H), 7.00 (t, J =8.8 Hz, 1H), 6.89 (s, 1H), 3.09 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (125.7 MHz,

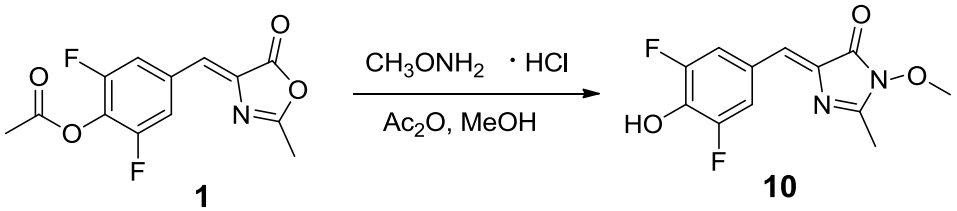
CD_3OD) δ 15.29 (CH_3), 26.82 (CH_3), 118.63 (d, J = 3.12Hz, CH), 120.04(CH), 120.20 (CH), 127.58 (d, J = 7.11Hz, CH), 130.97 (d, J = 2.7Hz), 138.30, 149.0 (d, J = 13.3Hz), 152.69 (d, J = 240.64Hz), 164.53, 172.25; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.06 min; purity, 95%; 235.37 $[M+H]^+$.

Synthesis of (*Z*)-4-(3,5-difluoro-4-hydroxybenzylidene)-1-hydroxy-2-methyl-1*H*-imidazol-5(4*H*)-one (**9**, DFHBI-1HO)[2]:



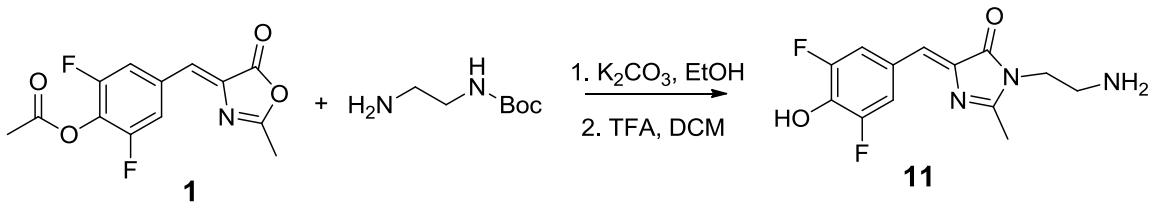
Compound **9-10** was synthesized as described previously.[2] Compound **1** (70 mg, 0.25 mmol) was refluxed with 1.0 ml of methanol, 26 mg (0.375 mmol) of hydroxylamine hydrochloride, and 31 mg (0.375) of sodium acetate for 6 h. The reaction mixture was cooled and the yellow precipitate was collected, washed with water and recrystallized from ethanol afford 32 mg (yield 50%) of **9** as a bright yellow solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.00(s, 1H), 7.96 (d, J = 9.3, 2H), 6.94 (s, 1H), 2.31 (s, 3H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 13.76 (CH_3), 116.67 (dd, J = 16.38Hz, 6.25Hz, CH×2), 125.79 (t, J = 9.24Hz), 127.30 (CH), 136.46, 138.10 (t, J = 16.3Hz), 153.54 (dd, J = 242.07Hz, 7.13Hz), 163.21, 167.34; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.12 min; purity, 95%; 255.08 $[M+H]^+$.

Synthesis of (*Z*)-4-(3,5-difluoro-4-hydroxybenzylidene)-1-methoxy-2-methyl-1*H*-imidazol-5(4*H*)-one (**10**, DFHBI-1MO):



Compound **1** (70 mg, 0.25 mmol) was refluxed with 1.0 ml of methanol, 23 mg (0.375 mmol) of methoxylamine hydrochloride, and 31 mg (0.375 mmol) of sodium acetate for 6 h. The reaction mixture was cooled and the yellow precipitate was collected , washed with water and recrystallized from ethanol afford 40 mg (yield 60%) of **10** as a bright yellow solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.97 (d, J = 9.45, 2H), 6.98 (s, 1H), 3.94 (s, 3H), 2.39 (s, 3H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 13.74 (CH_3), 65.24 (CH_3), 115.63 (dd, J = 16.36Hz, 6.25Hz, CH×2), 124.12 (t, J = 9.27Hz), 124.94 (CH), 134.43, 136.28 (t, J = 16.45Hz), 151.82 (dd, J = 241.33Hz, 7.5Hz), 160.38, 163.83; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.38 min; purity, 95%; 269.07 $[M+H]^+$.

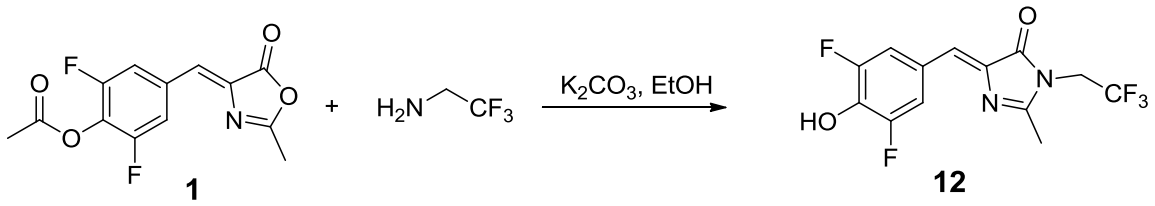
Synthesis of (*Z*)-1-(2-aminoethyl)-4-(3,5-difluoro-4-hydroxybenzylidene)-2-methyl-1*H*-imidazol-5(4*H*)-one (**11**, DFHBI-1AE):



Compound **1** (920 mg, 3.3 mmol) was refluxed with 14 ml of ethanol, 1.0 g (6.24 mmol) of N-Boc-ethylenediamine, and 1.26 g of potassium carbonate for 4 h. The reaction mixture was removed from heat and cooled to room temperature. The solvent was evaporated and the mixture was redissolved in a 1:1 mixture of ethyl acetate and 500 mM sodium acetate pH 3.0. The organic layer was separated, dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure and the reaction mixture was purified by column chromatography (MeOH: CH_2Cl_2 = 1: 20) to afford 1.1 g (yield 88%) of yellow solid. The result product (210mg) was redissolved in a 1:1 mixture of trifluoroacetic acid : dichloromethane and stirred at 25 ℃ for 2 h. After removal of solvent in vacuo, the reaction mixture were purified by silica gel chromatography (CH_2Cl_2:MeOH = 10:1) to afford 135 mg (yield 87%) of **11** as brown solid: ^{1}H NMR (500 MHz, CD_3OD) δ 7.80 (d, J = 9.0, 2H), 6.95 (s, 1H), 3.96 (t, J = 5.95, 2H), 3.23 (t, J = 5.95, 2H), 2.43 (s, 3H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 15.42 (CH_3), 39.46 (CH_2),

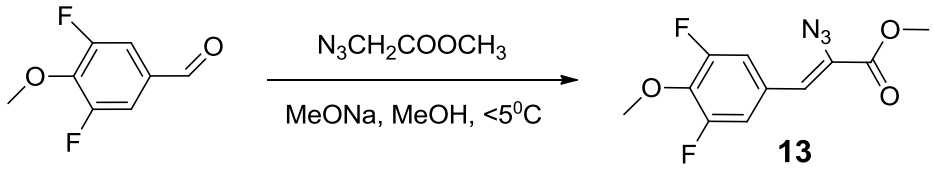
40.12 (CH_2), 116.46 (dd, J = 16.36Hz, 6.58Hz, CH×2), 125.97 (t, J = 8.91Hz), 126.76 (CH), 138.02, 138.85, 153.57 (dd, J = 241.85Hz, 7.2Hz), 164.51, 169.63; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 1.29 min; purity, 95%; 282.26 $[M+H]^+$; HRMS m/z: 282.1056 found (calcd. For $C_{13}H_{14}F_2N_3O_2$, $[M+H]^+$ 282.1054).

Synthesis of (*Z*)-4-(3,5-difluoro-4-hydroxybenzylidene)-2-methyl-1-(2,2,2-trifluoroethyl) -1*H*-imidazol-5(4*H*)-one (**12**, DFHBI-1T):



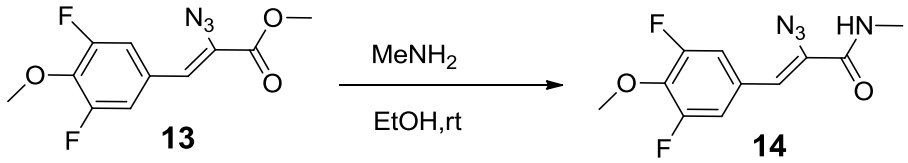
Compound **1** (472 mg, 1.68 mmol) was refluxed with 4 ml of ethanol, 250 mg (2.52 mmol) of 2,2,2-Trifluoroethylamine, and 348 mg of potassium carbonate for 4 h. The reaction mixture was removed from heat and cooled to room temperature. The solvent was evaporated and the mixture was redissolved in a 1:1 mixture of ethyl acetate and 500 mM sodium acetate pH 3.0. The organic layer was separated, dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure and the reaction mixture was purified by column chromatography (CH_2Cl_2: MeOH = 10:1) to afford 215 mg (yield 40%) of **12** as yellow solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.06 (s,1H), 7.99 (d, J = 9.6, 2H), 7.04 (s, 1H), 4.57 (q, J = 9.25, 2H), 2.42 (s, 3H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 42.45 (q, J = 35.51Hz, CH_2), 116.61 (dd, J = 16.24Hz, 6.59Hz, CH×2), 125.06 (sF_3, q, J = 278.76Hz), 125.65 (t, J = 9.24Hz), 127.50 (CH), 138.05, 138.42(t, J = 16.14Hz), 153.61 (dd, J = 241.80Hz, 7.2Hz), 162.97, 171.09; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 2.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.41 min; purity, 95%; 319.15 $[M-H]^-$ HRMS m/z: 319.0515 found (calcd. for $C_{13}H_8F_5N_2O_2$, $[M-H]^-$ 319.0506).

Synthesis of (*Z*)-methyl 2-azido-3-(3,5-difluoro-4-methoxyphenyl)acrylate (**13**)[3]



The synthesis of compound **13** is based on the synthesis of 2-Azido-3-(4-methoxyphenyl)acrylic acid methyl ester by Gim and colleagues.[3] To a solution of sodium methoxide (30 % w/w, 2.15 ml, 11.62 mmol) in dry methanol (4 mL) was added dropwise 3,5-difluoro-4-methoxybenzaldehyde (500 mg, 2.9 mmol) and methyl azidoacetate (1.34 g, 11.62 mmol) in methanol (1 mL) at -8 ℃. After stirring the suspension below 5 ℃ for 3 h, and then quenched by addition of ice water. The resulting mixture was stirred for 10 min, filtered and washed with water to afford the title compound **13** (484 mg, 62 %) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41 (d, J =9.7 Hz, 2H), 6.71 (s, 1H), 4.05 (s, 3H), 3.91 (s, 3H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 53.21 (CH_3), 61.89 (CH_3), 114.45 (dd, J = 18.1Hz, 6.25Hz, CH×2), 122.70, 126.36, 127.81 (t, J = 9.7Hz), 137.29 (t, J = 13.7Hz), 155.03 (dd, J = 247.3Hz, 6.8Hz), 163.73.

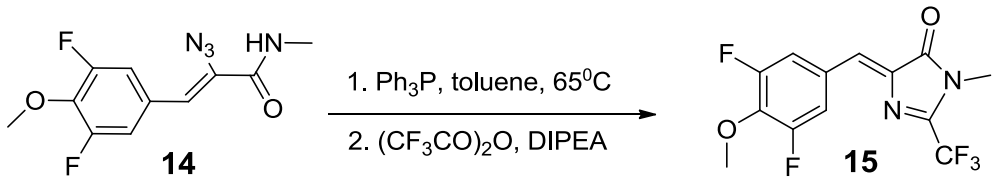
Synthesis of (*Z*)-2-azido-3-(3,5-difluoro-4-methoxyphenyl)-*N*-methylacrylamide (**14**)[4]



The synthesis of compound **14-16** is based on the synthesis of (*Z*)-4-(4-hydroxybenzylidene)-1-methyl-2-(trifluoromethyl)-1*H*-imidazol-5(4*H*)-one by Baranov and colleagues.[4] A solution of (*Z*)-methyl 2-azido-3-(3,5-difluoro-4-methoxyphenyl)acrylate **13** (410 mg, 1.49 mmol) and aqueous methylamine (0.92 ml, 40%) in ethanol (5 ml) was stirred at room temperature for 24 h. Reaction mixture was evaporated, crude product dissolved in 15 ml of chloroform, washed with aqueous HCl (5%, 6 ml), water (2×5 ml) and dried over sodium sulfate. The solution was concentrated in vacuo to give 384 mg product **14** (yield 96%) as light yellow solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23 (d, J =9.5 Hz, 2H), 6.36 (s, 1H), 4.04 (s, 3H), 2.96 (d, J =4.9 Hz, 3H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 26.25 (CH_3), 61.80 (CH_3), 113.39 (dd, J =

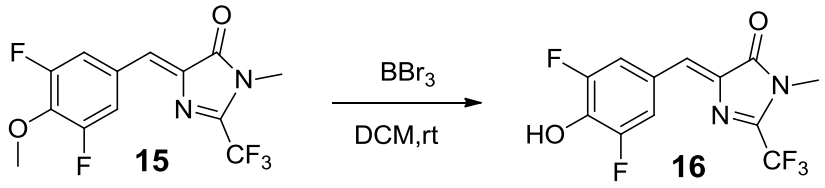
18.4Hz, 5.8Hz, CH×2), 115.77, 128.84 (t, J = 9.7Hz), 131.15, 135.50 (t, J = 13.7Hz), 154.45 (dd, J = 238.7Hz, 6.8Hz), 163.35.

Synthesis of (*Z*)-4-(3,5-difluoro-4-methoxybenzylidene)-1-methyl-2-(trifluoromethyl)-1*H*-imidazol-5(4*H*)-one (**15**)



A solution of amide **14** (380 mg, 1.42 mmol) and triphenylphosphine (409 mg, 1.56 mmol) in dry toluene (7 ml) was heated to 65 ℃ under argon. Yellow precipitate formed and effervescence appeared. After 30 minutes the reaction mixture was cooled to room temperature and 2,2,2-trifluoroacetic anhydride (0.4 ml, 2.84 mmol) and DIPEA (0.247 ml, 1.42 mmol) was added. The mixture was heated to 40 ℃ for 1.5 h. The reaction mixture was cooled and diluted with chloroform (15 ml), washed with saturated $NaHCO_3$ solution (15 ml), water (2 × 8 ml), brine (2 × 8 ml) and dried over sodium sulfate. The solvent was evaporated and the product was purified by column chromatography (EtOAc : hexane = 2:4) to afford 120mg of **15** (yield 26%) as yellowish solid: ^{1}H NMR (500 MHz, CD_3OD) δ 7.79 (d, J =9.5 Hz, 2H), 7.20 (s, 1H), 4.04 (s, 3H), 3.30 (s, 3H).

Synthesis of (*Z*)-4-(3,5-difluoro-4-hydroxybenzylidene)-1-methyl-2-(trifluoromethyl)-1*H*-imidazol-5(4*H*)-one (**16**, DFHBI-2T)



A solution of (*Z*)-4-(3,5-difluoro-4-methoxybenzylidene)-1-methyl-2-(trifluoromethyl)-1*H*-imidazol-5(4*H*)-one **15** (64 mg, 0.2mmol) in dry dichloromethane (0.5 ml) was cooled to 0 ℃ and the solution of boron tribromide in dichloromethane (1M, 0.3 ml) was added. The mixture was kept at room temperature for 15 minutes and after that 5 ml of dichloromethane and 2 ml of water was added. The organic layer was washed with

saturated $NaHCO_3$ solution (5 ml), water (2 ×5 ml) and dried over sodium sulfate. The solvent was evaporated and the product was purified by column chromatography ($CHCl_3$: EtOH = 20:1) to afford 20 mg of **16** (yield 33%) as yellow solid: ^{1}H NMR (500 MHz, CD_3OD) δ 7.86 (d, J = 9.55, 2H), 7.31 (s, 1H), 3.31 (s, 3H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 27.56 (CH_3), 117.63 (dd, J = 16.35Hz, 6.52Hz, CH×2), 119.21 (sF_3, q, J = 272.24Hz), 124.96 (t, J = 9.16Hz), 134.09 (CH), 137.07, 139.72(t, J = 16.33Hz), 151.81 (q, J = 38.93Hz), 153.60 (dd, J = 242.68Hz, 6.2Hz), 170.52; LC/MS (LC: gradient 20-95% MeCN [0.1% HCO_2H] over 4.5 min, 0.5 ml/min flow rate, MS: ESI+): retention time, 2.91 min; purity, 95%; 305.13 $[M-H]^-$ HRMS m/z: 305.0355 found (calcd. For $C_{12}H_6F_5N_2O_2$, $[M-H]^-$ 305.0349).

C. Affinity measurements

Dissociation constants (K_D) for the Spinach2-fluorophore complexes were determined by measuring the increase in fluorescence as a function of increasing fluorophore concentration in the presence of a fixed concentration of RNA aptamer as previously described.[1] For each concentration of fluorophore measured, a background signal for fluorophore alone was also measured and subtracted from the signal measured for RNA and fluorophore together. Curves were determined using a nonlinear regression analysis in Prism software and matched by least squares fitting to a standard dose-response model for 1:1 complexation.

D. Quantum yield measurements

All quantum yields were determined by comparing the integral of the corrected emission spectra for each fluorophore or Spinach2-fluorophore complex with the corresponding integral obtained from a reference solution of DFHBI or Spinach2-DFHBI with the same experimental parameters (excitation wavelength, slit widths, photomultiplier voltage etc.). The quantum yield (Φ) is then calculated as described previously[5]:

$$\Phi=\Phi_R\times\frac{\mathrm{Int}}{\mathrm{Int}_R}\times\frac{1\text{-}10^{A_R}}{1\text{-}10^{A}}\times\frac{n^2}{n_R^2}$$

Int is the area under the emission peak (on a wavelength scale), A is absorbance (also called "optical density") at the excitation wavelength, and n is the refractive index of the

solvent, the ratio is 1 for this study. The subscript R denotes the respective values of the reference substance. All measurements for RNA-fluorophore complexes were taken in the presence of excess RNA to avoid interference from unbound fluorophore. The absolute quantum yields for reference fluorophores were taken to be 0.0007 for DFHBI and 0.72 for Spinach2-DFHBI[1].

D. Fluorescence measurements of Spinach2-SAM sensor with different fluorophores

A solution of RNA sensor (2 μM) and fluorophore (10 μM) was incubated at 37°C in a buffer containing 40 mM HEPES pH 7.4, 125 mM KCl and 5 mM $MgCl_2$ as well as SAM (0 or 1 mM) for 30 min at ambient temperature. Fluorescence emission was recorded using the following instrumental parameters: $E_{x,\ DFHBI}$ = 460 nm, $E_{x,\ DFHBI\text{-}1T}$ = 482 nm, $E_{x,\ DFHBI\text{-}2T}$ = 500 nm; slit widths, 10 nm. The fluorescence intensity was then plotted against wavelength.

E. Mammalian cell imaging with different fluorophores

COS-7 cells were transiently transfected to express $p(CGG)_{60}$-Spinach2 as previously described.[6] At 24 h post-transfection, growth medium (DMEM supplemented with 10% fetal bovine serum [FBS]) was replaced with imaging medium (DMEM lacking phenol red supplemented with 10% FBS, 20 μM appropriate fluorophore, 25 mM HEPES pH 7.4, and 5 mM $MgSO_4$). Cells were cultured in imaging medium for 30 min prior to imaging. In order to image the same cells with different fluorophores, cells were imaged with one fluorophore and then changed into media lacking fluorophore for 30 min. After 30 min, this media was supplemented to 20 μM of the second fluorophore and incubated for an additional 30 min before imaging.

For imaging 5S-Spinach2 with DFHBI and DFHBI-1T, HEK-293T cells were transiently transfected with pAV5S-Spinach2 as previously described.[6] 36 h post-transfection, cells were incubated with imaging media containing 20 μM of appropriate fluorophore for 30 min prioir to image acquisition.

In all cases, live fluorescence images were taken with a CoolSnap HQ2 CCD camera through a 60X oil objective (Plan Apo 1.4 NA) mounted on a Nikon TE2000 epifluorescence microscope using FITC or YFP filter sets and analyzed with the NIS-Elements software.

References

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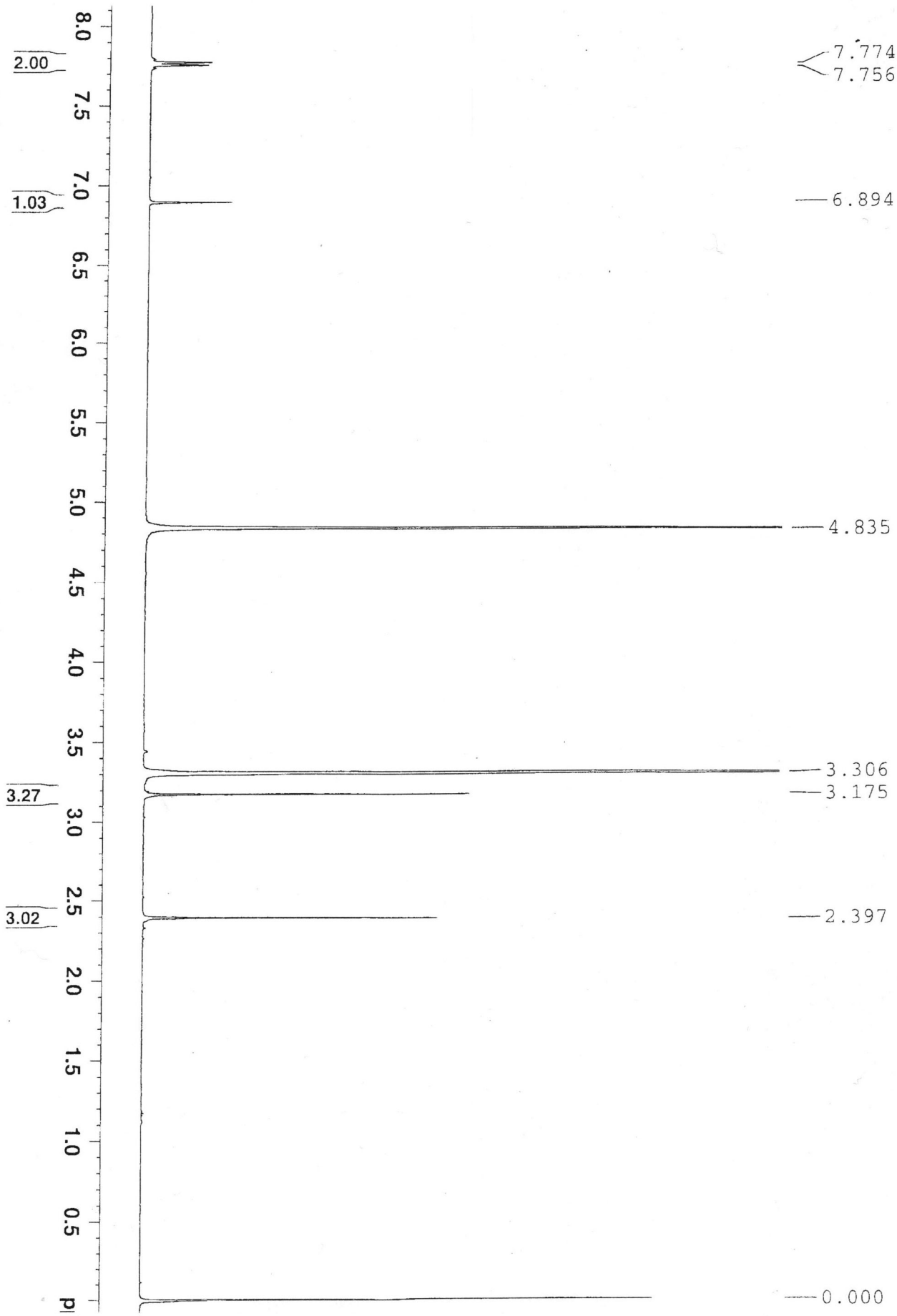
(4) Baranov, M. S.; Solntsev, K. M.; Lukyanov, K. A.; Yampolsky, I. V. *Chem Commun (Camb)* **2013**, *49*, 5778.

(5) *Brouwer, A. M. Pure Appl. Chem*. **2011**, *83*, 2213.

(6) Strack, R. L.; Disney, M. D.; Jaffrey, S. R. *Nat Methods* **2013**, *10*, 1219.

DFHBI (1H-NMR)


8.0
7.5
7.0
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6.0
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5.0
4.5
4.0
3.5
3.0
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2.0
1.5
1.0
0.5
ppm
2.00
1.03
3.27
3.02
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7.756
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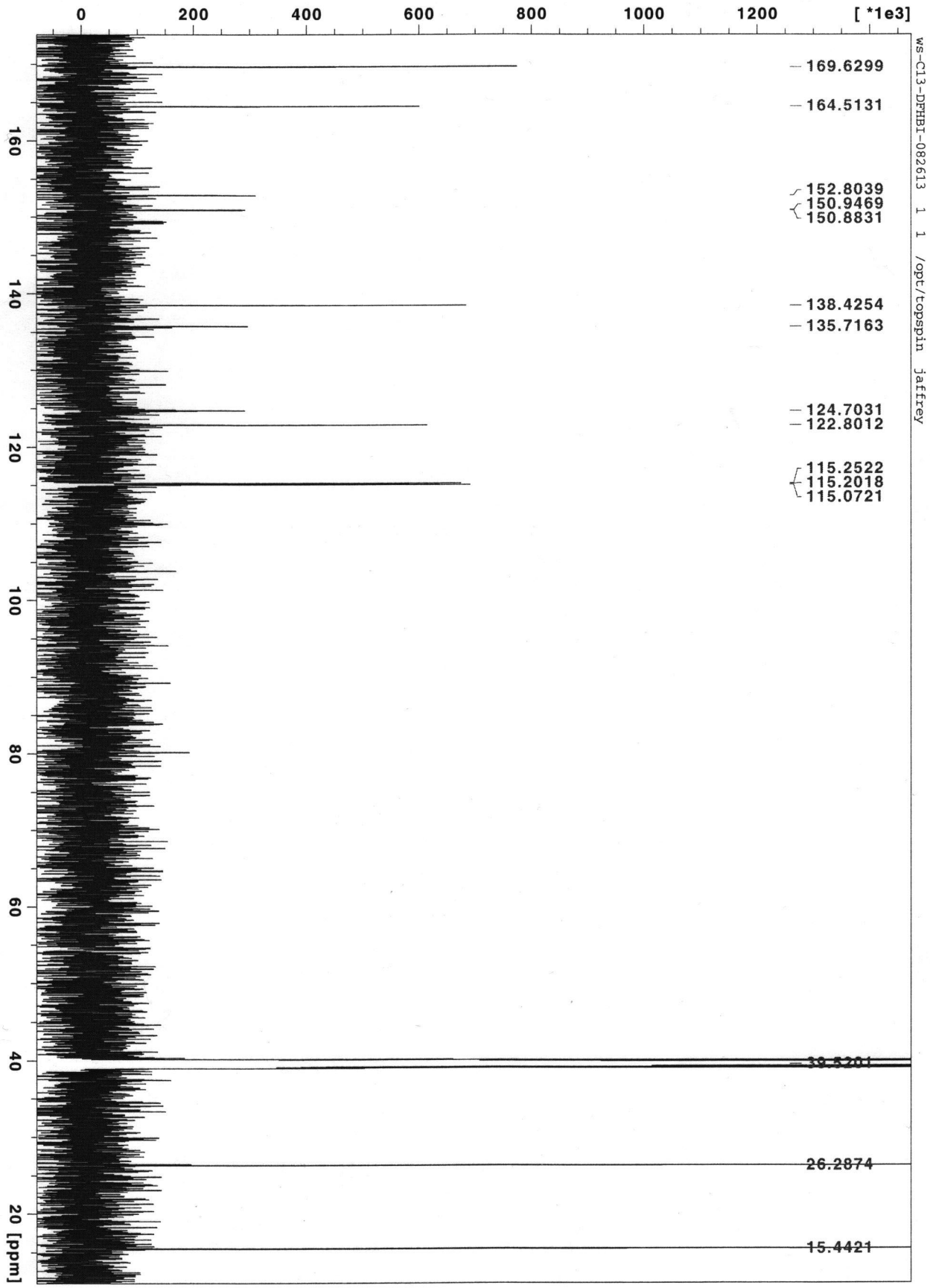
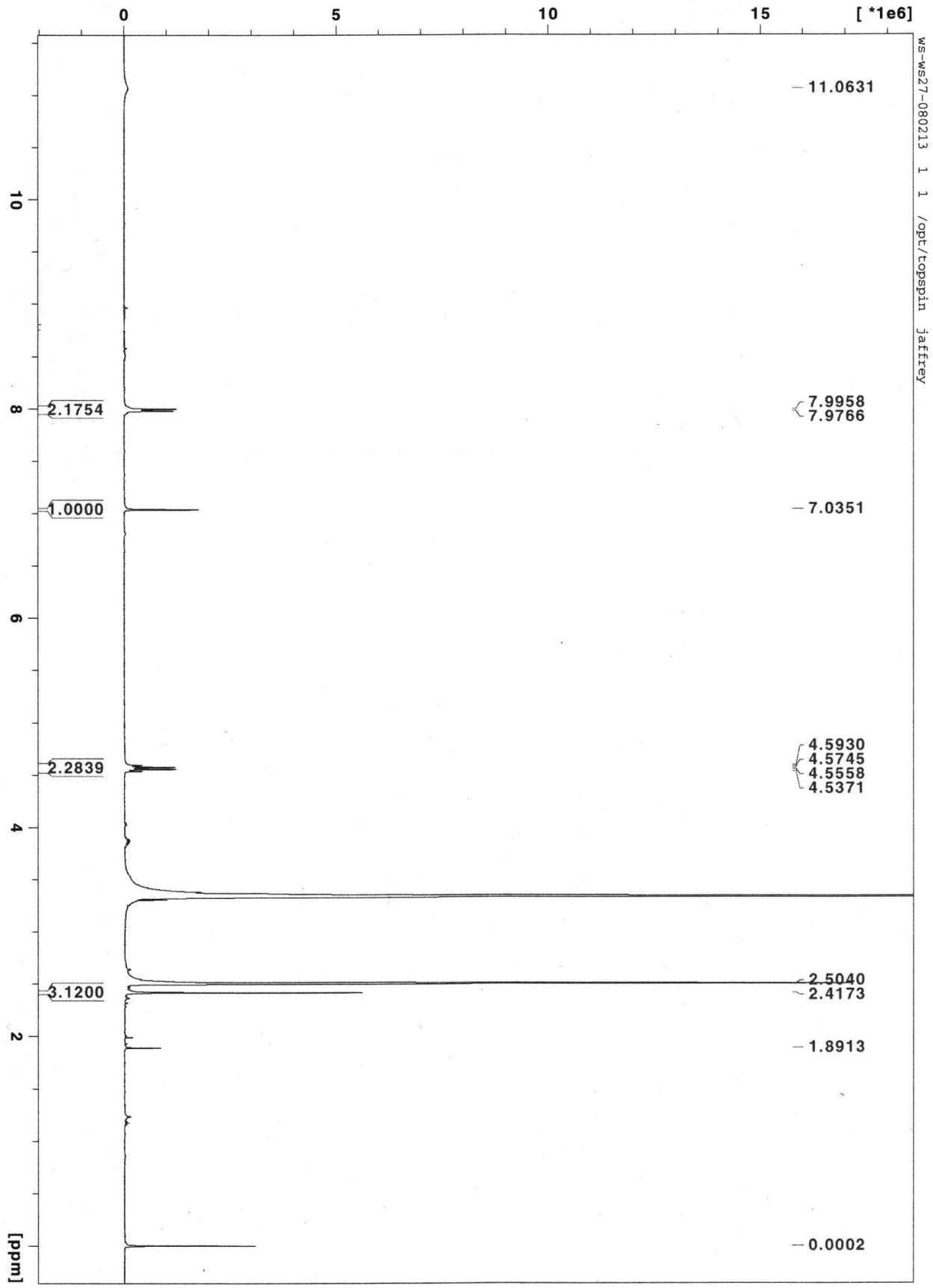
4.835
3.306
3.175
2.397
0.000

DFHBI (13C- NMR)



DFHBI-1T (1H-NMR)



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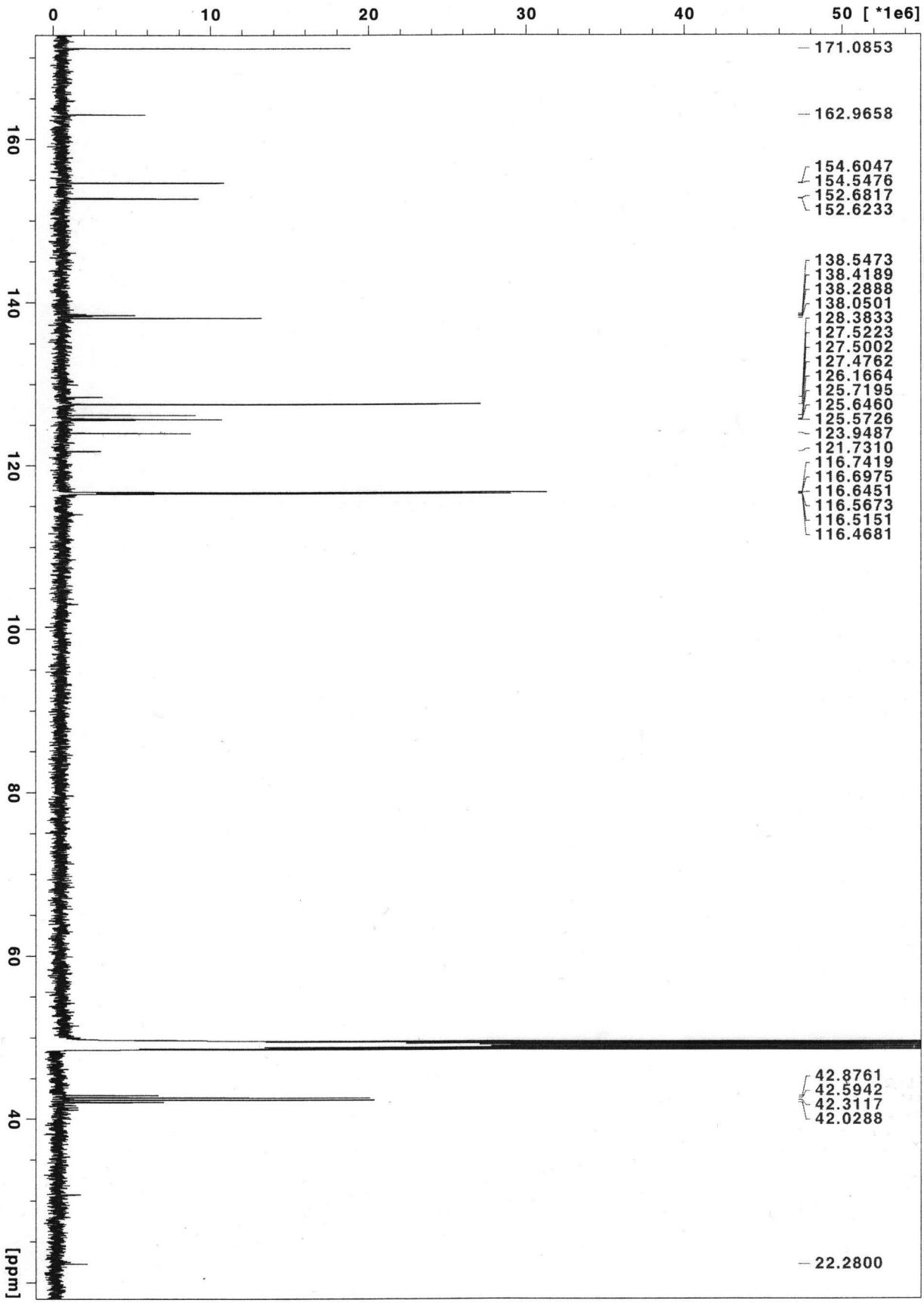

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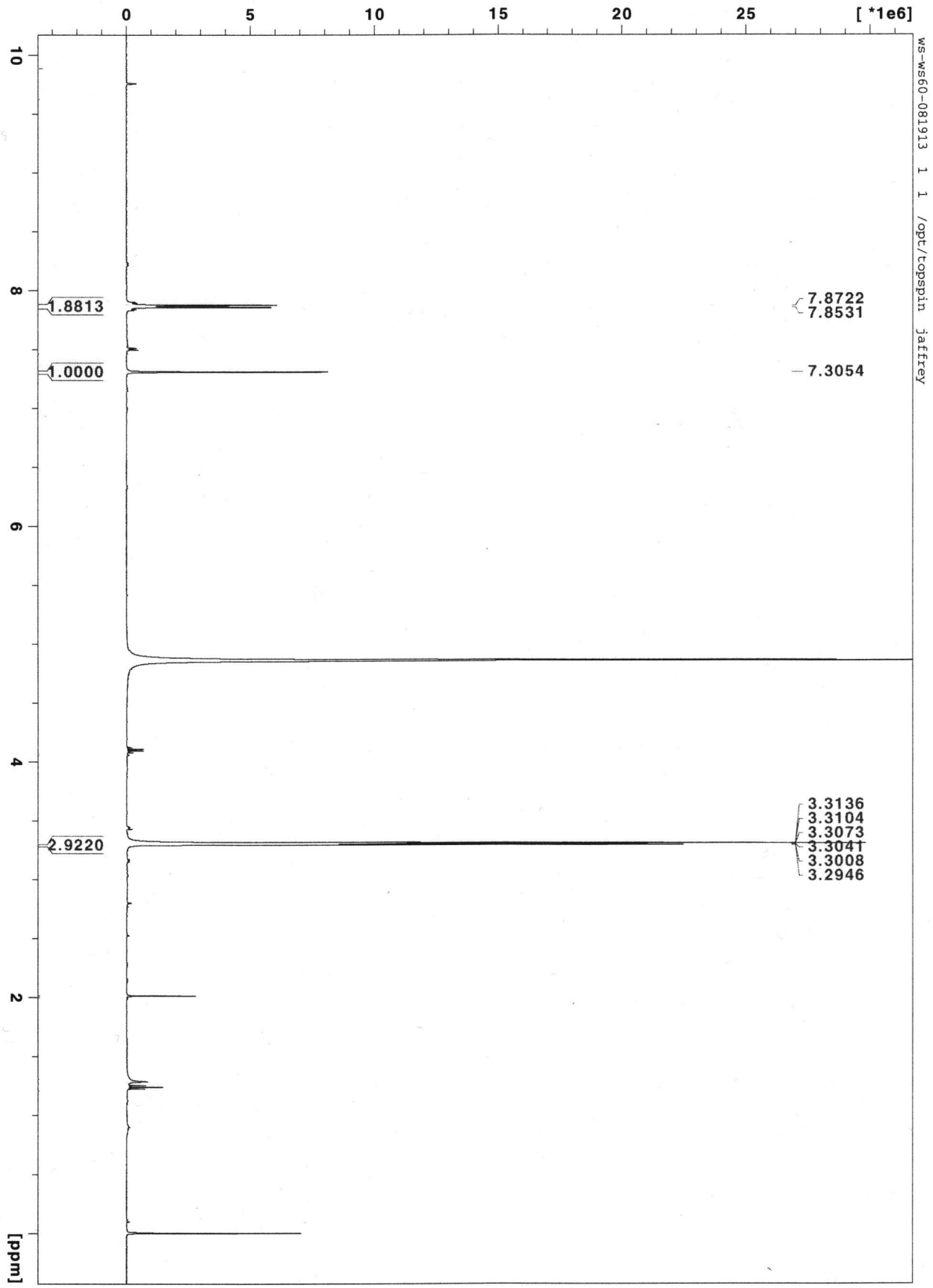

DFHBI-2T (13C-NMR)