Supporting Information for:

Synthesis of New Fluorogenic Cyanine Dyes and Incorporation into RNA Fluoromodules

Tudor P. Constantin,[†] Gloria L. Silva,[†] Kelly L. Robertson,[†] Tamara P. Hamilton,[†] Kaitlin Fague,[†] Alan S. Waggoner,[§] and Bruce A. Armitage[†§*]

Experimental Section

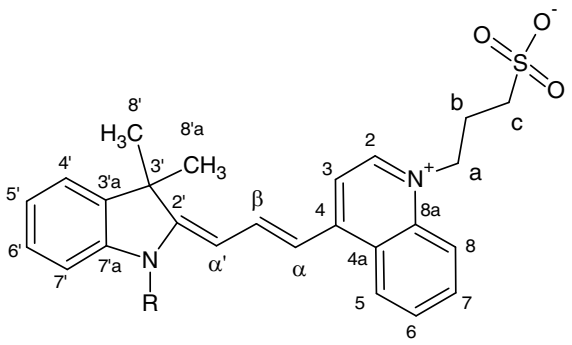
Materials and Methods

The ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz or 500 MHz and at 75.47 or 125.76 MHz, respectively, on a Bruker Avance using MeOD-d3 or DMSO-d6 as solvent. The 1H and 13C signals for DIR and compound 4 were assigned by a combination of COSY, long range COSY, HMBC and HSQC experiments. (Spectra are shown beginning on page 13.) The electrospray ionization mass spectrometry (ESI-MS) experiments were run on a Finnigan LCQ quadrupole ion trap mass spectrometer using Xcalibur Ver.1.2.

Mass spectral data was collected at the CUNY Mass Spectrometry Facility at Hunter College on an Agilent Technologies 6520 high resolution Q-TOF mass spectrometer attached to an Agilent Technologies 1200 HPLC system. The sample was ionized by electrospray ionization in the negative mode. The sample was injected into the instrument in a solution of methanol containing 0.1% formic acid at a flow rate of 200ul/min. Instrument parameters were as follows: fragmentor=165 volts, drying gas temperature=300 deg C, drying gas flow = 12 L/min, nebulizer pressure = 40psi, capillary voltage = 3500V, scan range = 118-3000 m/z, reference masses used were m/z=112.985587 and m/z=966.000725. Experiments involving ms/ms used a ramped collision energy with an offset of 10 volts and a slope of 2 volts per 100 m/z. Funding for this instrument system was provided by NIH Shared Instrumentation Grant 1S10RR022649-01 and The City University of New York.

Chromatographic separations were performed on reversed phase C18 silica gel (Separation Method Technologies, Inc., DE) and high performance liquid chromatography (HPLC) was done on a Waters system using a C18 Symmetry column 300A, 7 μm particle size, 19 x 150 mm. Chemicals were purchased from Sigma-Aldrich (USA); O-(7-Azabenzotriazole-1-yl)-N,N,N'N'-tetramethyluronium hexafluorophosphate (HATU) was purchased from Applied Biosystems (CA).

Synthesis of Dimethylindole Red and Biotinylated Analogue



R = -CH_3 (**DIR**)
a'

R= -CH_2-CH_2-CH_2-CH_2-CH_2-COO^- (**4**)
a' b' c' d' e' f'

*Dimethylindole Red (**DIR**)*

3-(4-Methylquinolinium-1-yl)propane-1-sulfonate (**1**) was synthesized by mixing lepidine (1.54 g, 10.7 mmol) with propanesultone (1.25 g, 10.2 mmol) and heating at 110 °C for 1.5 h. The mixture hardened almost immediately. After cooling, the solid was crushed and washed several times with ethyl ether, dried under vacuum and gave satisfactory ^{1}H NMR; 92% yield (2.92 g, 9.9 mmol). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.39 (1H, d, *J* = 6.0); 8.71 (1H, d, *J* = 9.0 Hz); 8.55 (1H, dd, *J* = 8.4; 1.2 Hz); 8.27 (1H, ddd, *J* = 9.1; 7.0; 1.5 Hz); 8.09-8.02 (2H, m); 5.18 (2H, t, *J* = 7.2 Hz); 3.01 (3H, s); 2.55 (2H, t, *J* = 7.2 Hz); 2.28 (2H, br quint, *J* = 7.2 Hz).

The hemidye **2** was prepared by reacting (**1**) (1.0 g; 3.8 mmol) with *N,N'*-diphenylformamidine (740.0 mg; 3.8 mmol) at 160 °C for 30 min.[1] (This procedure rendered less by-products and gave higher yields than reactions using solvents.) The resulting dark brown solid was crushed and washed several times with ethyl ether. The dry powder was crystallized from ethanol (50 mL) to give 4-(2-anilinovinyl)-3-sulfopropenyl-quinolinium (**2**) in fair purity (538.0 mg; 1.5 mmol; 40% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.98 (1H, d, *J* = 12.5); 8.89 (1H, t, *J* = 12.5); 8.68 (1H, d, *J* = 7.0 Hz); 8.33 (2H, br d, *J* = 9.0 Hz); 8.14 (1H, d, *J* = 7.0 Hz); 8.05 (1H, br t, *J* = 8.0); 7.80 (1H, br t, *J* = 8.0 Hz); 7.50-7.38 (3H, m); 7.14 (1H, t t, *J* = 8.0; 1.5 Hz); 6.75 (1H, d, *J* = 12.5 Hz); 4.81 (2H, t, *J* = 7.3 Hz); 2.53 (2H, t, *J* = 7.3 Hz); 2.18 (2H, quint, *J* = 7.3 Hz).

1-Methyl-2,3,3-trimethyl-3*H*-indolium iodide (29.0 mg, 0.097 mmol) and **2** (35.0 mg, 0.095 mmol) were dissolved in 1 mL of 9:1 (v/v) pyridine/DIEA in a round-bottom flask; 100 μL of acetic anhydride were added to this solution. The round bottom flask was slightly heated until the solution turned green, then it was wrapped in aluminum foil and kept in the dark for 4 hours. The crude dye was precipitated and washed exhaustively with ethyl ether. Dimethylindole red (**DIR**) was purified by RP-C18 column chromatography using water/methanol mixtures as eluent. Upon evaporation of the methanol, **DIR** crystallized from water and was obtained as shiny, yellow-reddish needles (10.0 mg, 0.022 mmol, 23% yield). UV-Vis λ_{max} 602.0 nm (MeOH), ε = 134,000 $M^{-1}cm^{-1}$; ϕ_f = 0.15 (90% glycerol; 24 °C); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.65 (1H, d, *J* = 7.8 Hz, H-2); 8.60 (1H, d, *J* = 7.0 Hz, H-5); 8.34 (1H, t, *J* = 13.1 Hz, H-β); 8.33 (1H, d, *J* = 8.5 Hz, H-8); 8.11 (1H, d, *J* = 7.0 Hz, H-3); 8.03 (1H, t, *J* = 7.5 Hz, H-7); 7.79 (1H, t, *J* = 8.0 Hz, H-6); 7.50 (1H, d, *J* = 7.5 Hz, H-4'); 7.32 (1H, t, *J* = 7.5 Hz, H-6'); 7.28 (1H, d, *J* = 13.0 Hz, H-α); 7.18 (1H, d, *J* = 7.5 Hz, H-7'); 7.09 (1H, t, *J* = 7.5 Hz, H-5'); 6.20 (1H, d, *J* = 13.0 Hz, H-α'); 4.85 (2H, t, *J* = 7.0 Hz, H-a); 3.45 (3H, br s, H-a'); 2.52 (2H, t, *J* = 7.0 Hz, H-c); 2.18 (2H, quint, *J* = 7.0 Hz, H-b); 1.68 (6H, br s, H-8'and H-8'a); ^{13}C NMR (75.47 MHz) in ppm: 26.1 (C-b); 28.6 (C-8', C-8'a); 30.5 (C-a'); 47.9 (C-3', C-c); 54.0 (C-a); 100.6 (C-α'); 109.7 (C-7'); 112.0 (C-α); 113.0 (C-3); 118.9 (C-8); 122.5 (C-4'); 123.1 (C-5'); 125.3 (C-4a); 126.0 (C-5); 127.8 (C-6); 128.6 (C-6'); 134.3 (C-7); 138.4 (C-8a); 140.5 (C-3'a); 143.5 (C-7'a); 143.9 (C-2); 144.5 (C-β); 152.2 (C-4); 168.7 (C-2'). ESI-MS (positive mode) *m/z* 449.21($M+H^+$), calculated for $C_{26}H_{29}N_2O_3S$ *m/z* 449.58. High resolution MS: 448. 18247 (calc. for $C_{26}H_{29}N_2O_3S$: 448. 18206)

DIR-Biotin

1-(5-Carboxypentyl)-2,3,3-trimethylindolinium bromide (**3**) (189.0 mg; 0.5 mmol, provided by Dr. Brigitte Schmidt) was reacted with **2** (400.0 mg; 1 mmol) in 2 mL of 9:1 (v/v) pyridine/DIEA and 100 μL of acetic anhydride. The reaction was heated for a few seconds until the blue color became apparent. The reaction proceeded overnight at room temperature. The crude dye was precipitated and washed exhaustively with ethyl ether. The dye **4** was pre-purified on a RP-C18 column with mixtures of water/methanol of increasing strength. Pure dye was obtained by semi-prep HPLC using a gradient of 95% B at 5 min to 95% D at 35 min, flow rate 5 mL/min at 25 °C, solvents: 0.1% TFA in water (solvent B) and 0.1% TFA in acetonitrile (solvent D), t_r = 24 min. Counterion exchange was achieved by dissolving the dye into a 5% sodium bicarbonate solution and loading it onto a RP-C18 column pre-treated with the same solution. The dye was washed extensively with deionized water and eluted from the column with pure methanol. Yield 32% (102.0 mg; 0.18 mmol). UV-Vis λ_{max} 607.0 nm (MeOH), ε = 133,800 $M^{-1}cm^{-1}$; ϕ_f = 0.13 (90% glycerol; 29 °C); ^{1}H NMR (500 MHz, MeOD-d_3) δ: 8.60 (1H, d, *J* = 8.0

Hz, H-5); 8.50 (1H, d, *J* = 7.0 Hz, H-2); 8.43 (1H, t, *J* = 13.0 Hz, H-β); 8.23 (1H, d, *J* = 8.0 Hz, H-8); 8.02 (1H, t, *J* = 8.0 Hz, H-7); 7.80 (1H, d, *J* = 7.0 Hz, H-3); 7.76 (1H, t, *J* = 8.0 Hz, H-6); 7.41 (1H, d, *J* = 7.5 Hz, H-4'); 7.33 (1H, t, *J* = 7.5 Hz, H-6'); 7.21 (1H, d, *J* = 13.0 Hz, H-α); 7.12 (1H, t, *J* = 7.5 Hz, H-5'); 7.10 (1H, d, *J* = 7.5 Hz, H-7'); 6.24 (1H, d, *J* = 13.0 Hz, H-α'); 4.85 (2H, br t, H-a); 3.99 (2H, br t, H-a'); 2.93 (2H, t, *J* = 7.0 Hz, H-c); 2.40 (2H, br quint, H-b); 2.31 (2H, br t, H-e');1.82 (2H, m, H-b'); 1.73 (6H, s, H-8' and H-8'a); 1.71 (2H, m, H-d'); 1.51 (2H, m, H-c'); ^{13}C NMR (125.76 MHz) in ppm: 25.7 (C-d'); 26.1 (C-b); 26.9 (C-b'); 27.2 (C-c'); 28.8 (8';8'a); 34.2 (C-e'); 43.7 (C-a'); 48.5 (C-c); 49.0 (C-3'); 56.3 (C-a); 101.0 (C-α'); 110.2 (C-7'); 112.7 (C-α); 117.6 (C-3); 118.9 (C-8); 122.9 (C-4'); 124.0 (C-5'); 126.6 (C-4a); 126.7 (C-5); 128.2 (C-6); 129.2 (C-6'); 135.1 (C-7); 139.5 (C-8a); 141.3 (C-3'a); 143.9 (C-7'a); 144.2 (C-2); 144.9 (C-β); 154.2 (C-4); 170.1 (C-2'); 177.1 (C-f'). ESI-MS (negative mode), *m/z* 547.13 (M^-), calculated for $C_{31}H_{35}N_2SO_5$, *m/z* 547.69.

The biotinylated dye used for aptamer selections was prepared by reacting **4** (11.4 mg; 0.020 mmol) and a 1.1 molar excess of HATU (8.4 mg; 0.022 mmol) in DIEA (4.06 μl; 0.022 mmol) for 15 minutes at room temperature. A 1.1 molar excess of biotin ethylenediamine hydrobromide (8.1 mg; 0.022 mmol) was added to the reaction mixture and reacted for 24 hours; a conversion of approximately 79% was calculated by ^{1}H NMR on the crude material. A small amount of the product was purified by HPLC (t_r = 22 min) using the same method as for **4**. ESI-MS (positive mode) *m/z* 818.40 ($M+H^+$), calculated for $C_{43}H_{57}N_6O_6S_2$, *m/z* 818.10.

Optical Spectroscopy

The extinction coefficient of **DIR** was determined from the absorbance of known concentrations of dye, using the Beer-Lambert law. Because even crystallized dye samples can contain various amounts of salt, the purity of the dye was assessed using ^{1}H NMR and an internal standard. Briefly, the target dye and a precisely measured amount of a pure NMR internal standard (thiazole orange) were dissolved in the same NMR tube and the proton NMR spectrum of the mixture was recorded. By knowing the concentration of the internal standard and the ratio between the integrals of unambiguously assigned, non-overlapping peaks for the two dyes, the concentration of the target dye can be determined accurately. The standard deviation in extinction coefficient measured by this method was less than 4%.

The fluorescence behavior of 1.0 μM **DIR** or thiazole orange (TO) was examined in 10 mM sodium phosphate buffer (pH 7) with 100 mM sodium chloride; in presence of 100 μM CT-DNA; and in 90% glycerol solution. Samples were excited at 600 nm for **DIR** or 570 nm for TO.

Fluorescence Quantum Yield Determination

The quantum yield of **DIR** in 90% glycerol in water was determined relative to a standard with a known quantum yield (Cy5 in our case, with a quantum yield of 0.27 in PBS). The integrated emission of a series of 5 dye solutions (all with the absorbance smaller than 0.1) was plotted against the absorbance at the excitation wavelength for both the standard and the unknown dye. (A typical graph is shown in Figure S1.) The slope of the two plots was used to calculate the quantum yield of the unknown dye, using the formula

$$\Phi_x = \Phi_{st} * \frac{m_x}{m_{st}} * \left(\frac{n_x}{n_{st}}\right)^2$$

where,

Φ_x - fluorescence quantum yield of unknown dye;

Φ_{St} - fluorescence quantum yield of standard;

m_x - slope of integrated fluorescence vs. absorbance for the unknown dye;

m_{St} - slope of integrated fluorescence vs. absorbance for the standard;

n_x - refractive index of unknown dye's solvent;

n_{St} - refractive index of standard's solvent.

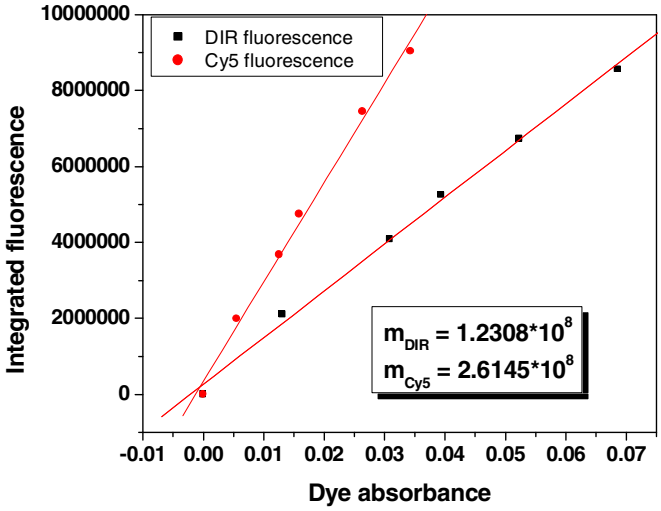


Figure S1. Fluorescence quantum yield determination.

Behavior of DIR in Cell Lysate

A potential future application of DIR is in intracellular labeling experiments, where the dye would bind to a genetically encoded RNA. In order for this to be feasible, the dye should not bind to other components of the cell nor be strongly fluorescent in the cytoplasm. To simulate such an environment for DIR, we prepared cell lysate using 10^5 HeLa cells, which were trypsinized and suspended in DMEM growth media. Cells were centrifuged, the medium was aspirated, and the cell pellet was washed once in PBS, then suspended in 500 uL PBS. An equal volume of glass beads was added and the cell suspension was vortexed for 3 minutes to break the cells. After centrifugation, the cell lysate was drawn off, DIR was added to a final concentration of 200 nM, and the fluorescence spectrum was recorded with excitation of DIR at 600 nm. The spectrum is shown in Figure S2, with DIR fluorescence in aqueous buffer and 90% glycerol shown for comparison. The fluorescence enhancement in lysate is 10-fold lower than in glycerol.

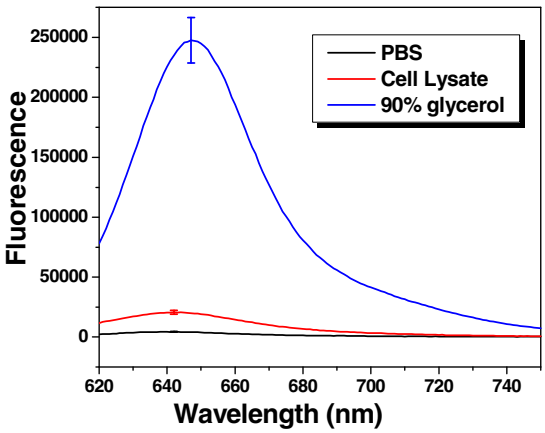


Figure S2. Fluorescence of DIR in HeLa cell lysate, phosphate-buffered saline (PBS) and 90% glycerol.

Aptamer Selection Protocol

The starting DNA pool and PCR primer sequences were identical to the ones described by Davis and Szostak.[2] Briefly, the DNA pool consists of 20 nucleotides (nts) of constant sequence at each end, flanking a central 64 nt region. Within this central region, there are two 26 nt randomized regions flanking a 12 nt constant sequence that is expected to fold into a stable stem-loop structure. The forward primer is 20 nucleotides in length, while the reverse primer is 40 nucleotides in length and consists of a primer binding region and a T7 RNA polymerase promoter. The sequences are:

Pool DNA

5'-GTGACGCGACTAGTTACGGA-N_{26}-CTGCCGAAGCAG-N_{26}-TTCATTCAGTTGGCGCCTCC-3'

(Underlined bases form the stem region of the hairpin.)

Forward Primer

5'-GTGACGCGACTAGTTACGGA-3'

Reverse Primer-T7 Promoter

5'-ATGTAATACGACTCACTATAGGAGGCGCCAACTGAATGAA-3'

The original DNA pool was PCR amplified (Taq DNApol, GeneChoice) using the following protocol: 30 sec at 95 °C, 30 sec at 54 °C, 2 min at 72 °C, repeat 12 times, followed by 5 min at 72 °C final elongation. For subsequent cycles, the PCR protocol was 30 sec at 95 °C, 30 sec at 54 °C, 40 sec at 72 °C, repeat 25 times. After PCR, the DNA product was verified on 1% agarose gels and then ethanol precipitated overnight.

The DNA template was *in vitro* transcribed with T7 RNApol (New England Biolabs) to yield the RNA pool complementary to the original DNA template. The initial pool consisted of approximately 10^{14} individual RNA sequences. After transcription, the RNA was extracted twice with phenol:chloroform, ethanol precipitated overnight and purified on 8% polyacrylamide gels. Typically, 40-90 μg of RNA were obtained.

Each RNA pool was annealed in selection buffer (5 mM $MgCl_2$, 150 mM KCl, 50 mM Tris, pH 7.4) and then used in the selection step. The selection step was performed on an affinity column containing the biotinylated **DIR** immobilized onto streptavidin-coated agarose beads (Sigma). For the first 3 cycles, a negative selection step was performed, in which the RNA pool was passed through a column containing just the streptavidin-coated agarose beads.

The RNA pool was incubated on the selection column for decreasing amounts of time, ranging from 4 hours in the first cycles to 15 minutes in the last cycles. After the incubation time has lapsed, the column was washed 3 times with selection buffer to remove the non-binding species, and then the winners were eluted by first suspending the agarose beads in 200 μl of selection buffer containing 500 μM free **DIR**. The column was heated to 70 °C and allowed to cool slowly to room temperature after which the flow-through was collected. This process was repeated two more times, the flow-through fractions were pooled and the RNA was ethanol precipitated. The recovered RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen), and the cDNA was PCR-amplified to start the next cycle. An aliquot of cDNA from each cycle was stored at -80 °C for future use.

DNA from cycle 15 was ligated into pUC2.1 plasmid and cloned in *E. coli* using a TA cloning kit (Invitrogen). Cloned plasmids were purified and tested by PCR for the presence of the insert. A total of 32 clones were tested for their ability to increase the fluorescence intensity of the dye (Figure 3 in paper). The sequence of the aptamer showing the highest fluorescence (**DIR-Apt1**) is given below (underline sequence corresponds to hairpin-forming constant element in original pool) and the lowest energy predicted secondary structure provided by mFold (M. Zuker) is shown in Figure S3.

5'–GGAGGCGCCAAGUGAAUGAAUCGGUAAUAAUUAGUUGGGUUAGU**CUGCUUC**
GGCAGGACCACUAAAAAAGAAACCCCUGCCUCCGUAACUAGUCGCGUCAC–3'

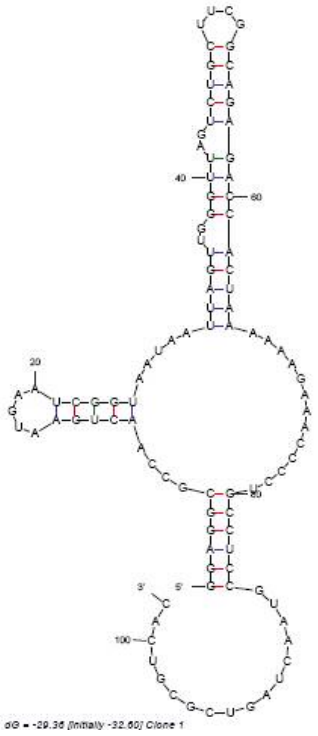


Figure S3. Predicted secondary structure of DIR-binding aptamer DIR-Apt1.

Fluorescence Enhancement Determination

RNA aptamers were transcribed from the corresponding DNA templates. The fluorescence intensity of a solution containing 200 nM **DIR** and 200 nM RNA aptamer was compared with a solution containing just 200 nM **DIR** (Figure 3 in paper).

For fluorescence in 90% glycerol measurements, one mL of 90% glycerol was heated to 60 °C in a plastic tube; the required amount of dye stock solution was added and mixed by repeatedly inverting the hot glycerol in the plastic tube. The solution was left to cool back to room temperature before it was transferred into a fluorescence cuvette and the fluorescence spectrum was recorded. This spectrum was compared with the fluorescence spectrum of a solution of 200 nM **DIR** in methanol.

Aptamer Cross-Reactivity

The **DIR**-**Apt1** aptamer and the previously described malachite green aptamer (obtained from Dharmacon, Inc.) were mixed with either **DIR** or malachite green, at final concentrations of 200 nM for both RNAs and dyes. Each sample was excited at the excitation maximum for the corresponding dye, the fluorescence spectrum was recorded and it was compared with the fluorescence spectrum of a 200 nM solution of the corresponding dye. As expected, the malachite green aptamer enhances the malachite green fluorescence while **DIR**-**Apt1** enhances the **DIR** fluorescence; however, the fluorescence of

malachite green in the presence of **DIR**-**Apt1**, and the fluorescence of **DIR** in the presence of the malachite green aptamer are not significantly enhanced (Figure S4).

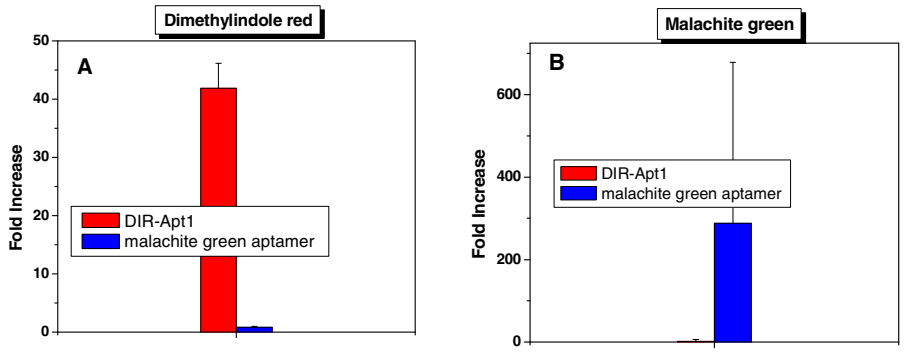


Figure S4. A: Fluorescence increase of **DIR** in the presence of **DIR**-**Apt1** or malachite green aptamer; B: Fluorescence of malachite green in the presence of **DIR**-**Apt1** or malachite green aptamer.

Binding Stoichiometry Determination

A continuous variation experiment was performed, in which the total concentration of the dye (**DIR**) + aptamer (**DIR**-**Apt1**) was kept constant at 0.1 μM, but the ratio of aptamer:dye was varied. The fluorescence intensity was recorded as a function of the **DIR** mole fraction. The maximum intensity was reached at a 1:1 dye : aptamer ratio. Data shown in Figure S5 are the result of triplicate experiments.

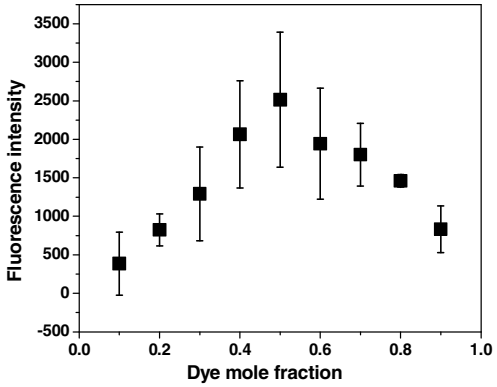


Figure S5. DIR:DIR-Apt1 binding stoichiometry determined by method of continuous variations. [RNA] + [**DIR**] = 100 nM.

Dissociation Constant Determination

Fluorescence titrations of **DIR** into a solution of **DIR-Apt1 RNA** in selection buffer were used to determine the dissociation constant of the aptamer to the dye.

$$K_d = \frac{[dye]_{free} * [RNA]_{free}}{[RNA - dye]}$$

$$F_\infty - F_0 \propto [dye]_{total}$$
$$F_i - F_0 \propto [RNA - dye]_i$$
$$\frac{F_\infty - F_0}{F_i - F_0} = \frac{[dye]_{total}}{[RNA - dye]_i}$$

$$[RNA - dye]_i = [dye]_{total} * \frac{F_i - F_0}{F_\infty - F_0}$$

$$[dye]_{free,i} = [dye]_{total} - [RNA - dye]_i = [dye]_{total} * \left(1 - \frac{F_i - F_0}{F_\infty - F_0}\right)$$

$$[RNA]_{free,i} = [RNA]_i - [RNA - dye]_i = [RNA]_i - [dye]_{total} * \frac{F_i - F_0}{F_\infty - F_0}$$

$$K_d = \frac{\left([dye]_{total}\left(1 - \frac{F_i - F_0}{F_\infty - F_0}\right)\right) * \left([RNA]_i - [dye]_{total} * \frac{F_i - F_0}{F_\infty - F_0}\right)}{[dye]_{total} * \frac{F_i - F_0}{F_\infty - F_0}}$$

$$K_d = \frac{\left(1 - \frac{F_i - F_0}{F_\infty - F_0}\right) * \left([RNA]_i - [dye]_{total} * \frac{F_i - F_0}{F_\infty - F_0}\right)}{\frac{F_i - F_0}{F_\infty - F_0}}$$

Solve this equation for $\frac{F_i - F_0}{F_\infty - F_0}$; let $\frac{F_i - F_0}{F_\infty - F_0}$ = x; let $[dye]_i$ = c; let $[RNA]_{total}$ = a.

Then, $K_d = \frac{(1-x) * (c - a * x)}{x}$

Or, $a * x^2 - (a + c + K_d) * x + c = 0$

The solutions of this quadratic equation are $x_{1,2} = \frac{(a + c + K_d) \pm \sqrt{(a + c + K_d)^2 - 4 * a * c}}{2 * a}$

which is

$$\frac{F_i - F_0}{F_\infty - F_0} = \frac{([dye]_{total} + [RNA]_i + K_d) \pm \sqrt{([dye]_{total} + [RNA]_i + K_d)^2 - 4 * [dye]_{total} * [RNA]_i}}{2 * [dye]_{total}}$$

Or

$$\frac{F_i - F_0}{F_\infty - F_0} * 2 * [dye]_{total} = ([dye]_{total} + [RNA]_i + K_d) \pm \sqrt{([dye]_{total} + [RNA]_i + K_d)^2 - 4 * [dye]_{total} * [RNA]_i}$$

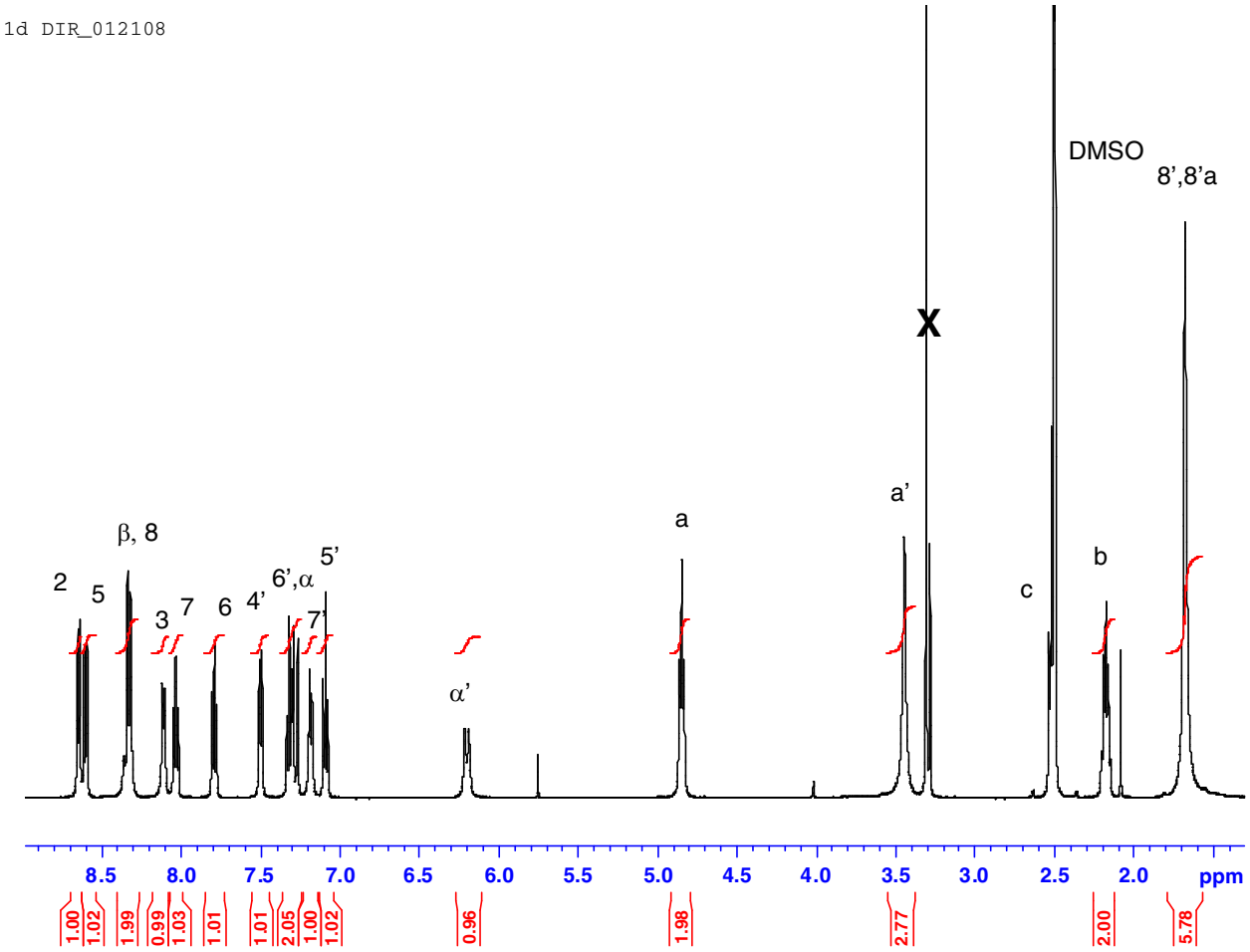
So plotting $\frac{F_i - F_0}{F_\infty - F_0} * 2 * [dye]_{total}$ as a function of $[RNA]_i$ and fitting it with the previous equation will yield the K_d for the RNA-dye binding.

References

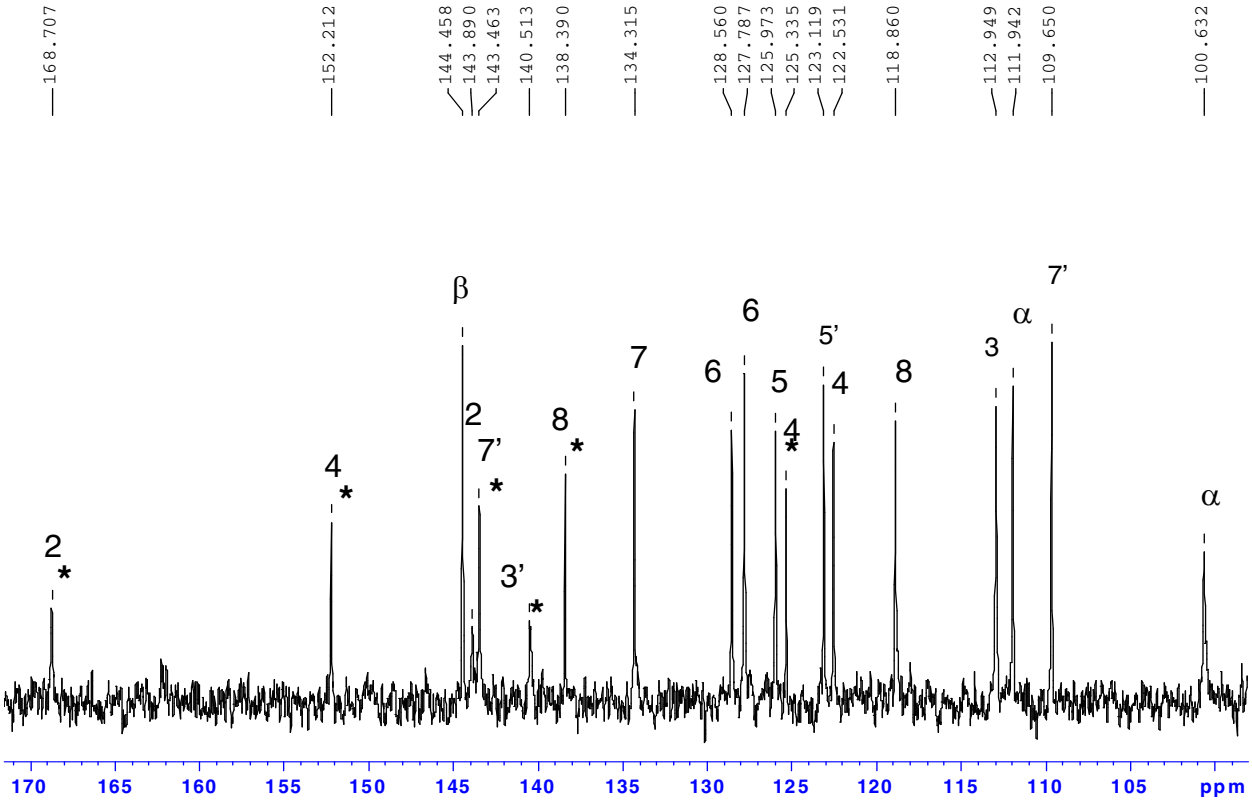
[1] Kishino, S.; Yasui, S.; Takahata, K.; Ohkawa, M. U.S. Patent 4,386,146. May 31, 1983, page 35.

[2] Davis, J. H.; Szostak, J. W. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11616-11622.

Acknowledgments. We thank the Molecular Biosensor and Imaging Center (MBIC-CMU) for providing intermediate **3** and Drs. Roberto Gil and Gayathri Withers for running the NMR experiments. The ESI mass spectra were recorded in the Center for Molecular Analysis, supported by NSF Grant No. DBI-9729351. NMR spectra were recorded in the CMU NMR facility, funded in part by NSF Grant No. CHE-9808188.

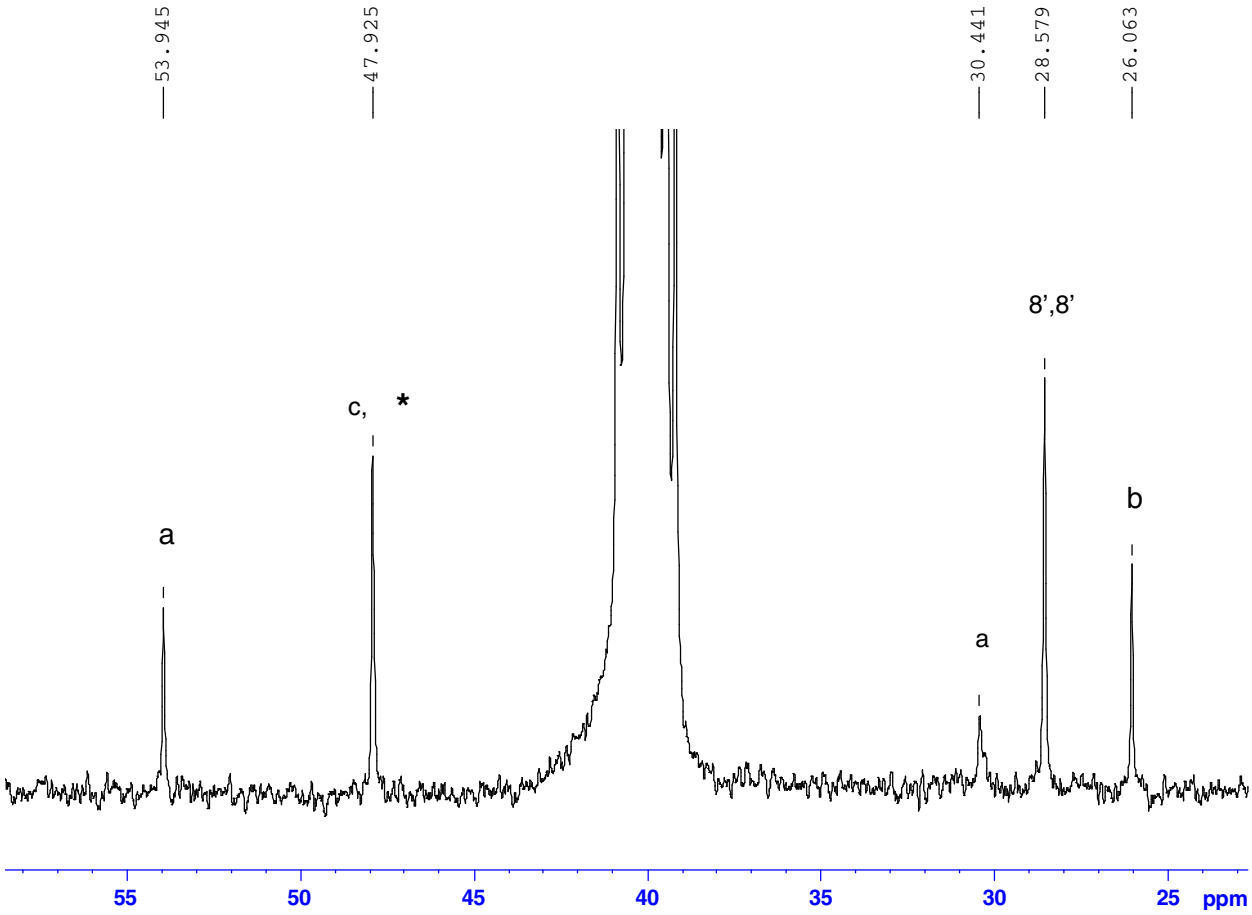


Spectrum 1: DIR: ^{1}H-NMR (500 MHz, DMSO-*d6*)



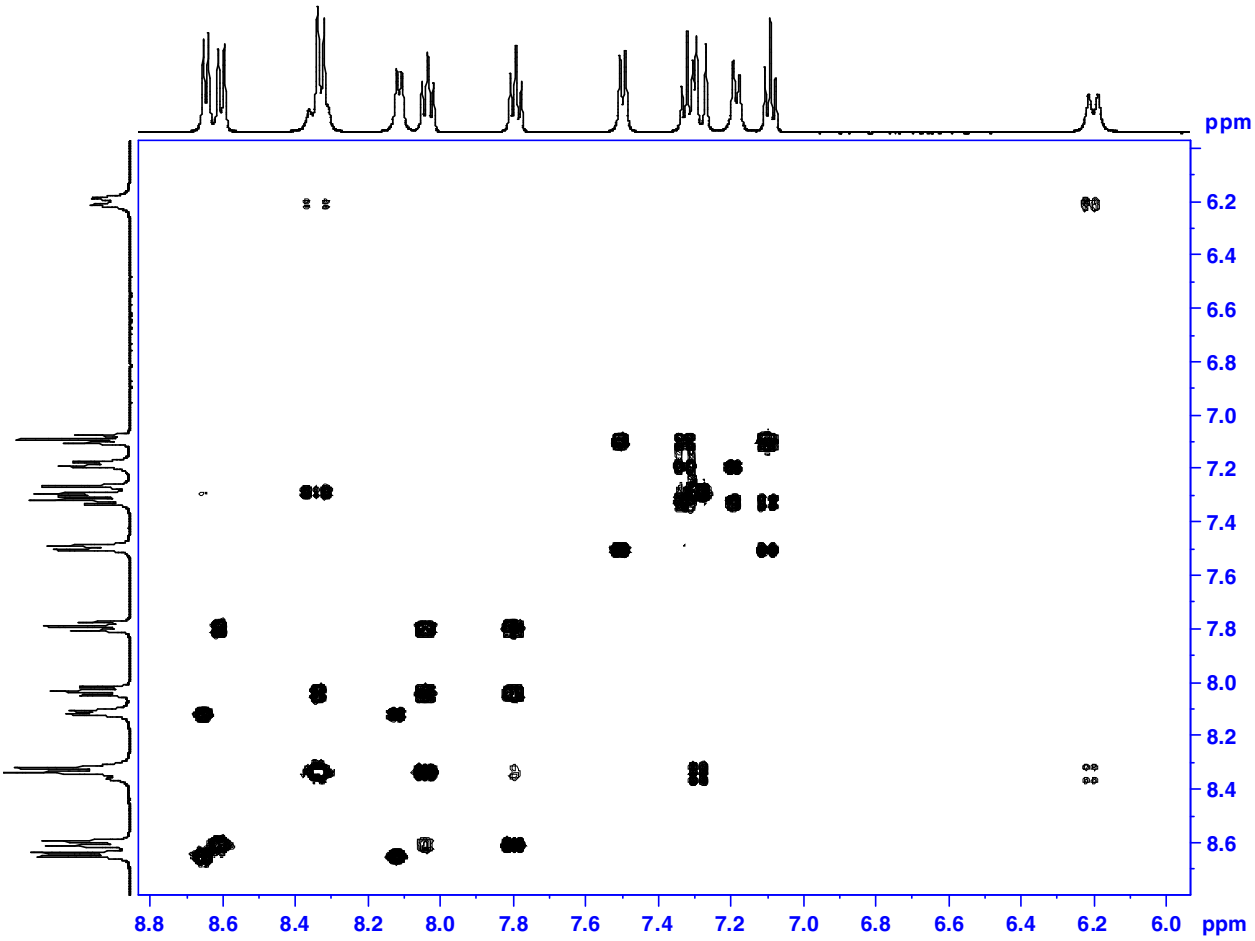
Spectrum 2: DIR ^{13}C NMR Expansion 1 (75.47 MHz, DMSO-*d6*)

* quaternary carbons, assigned by HMBC experiments

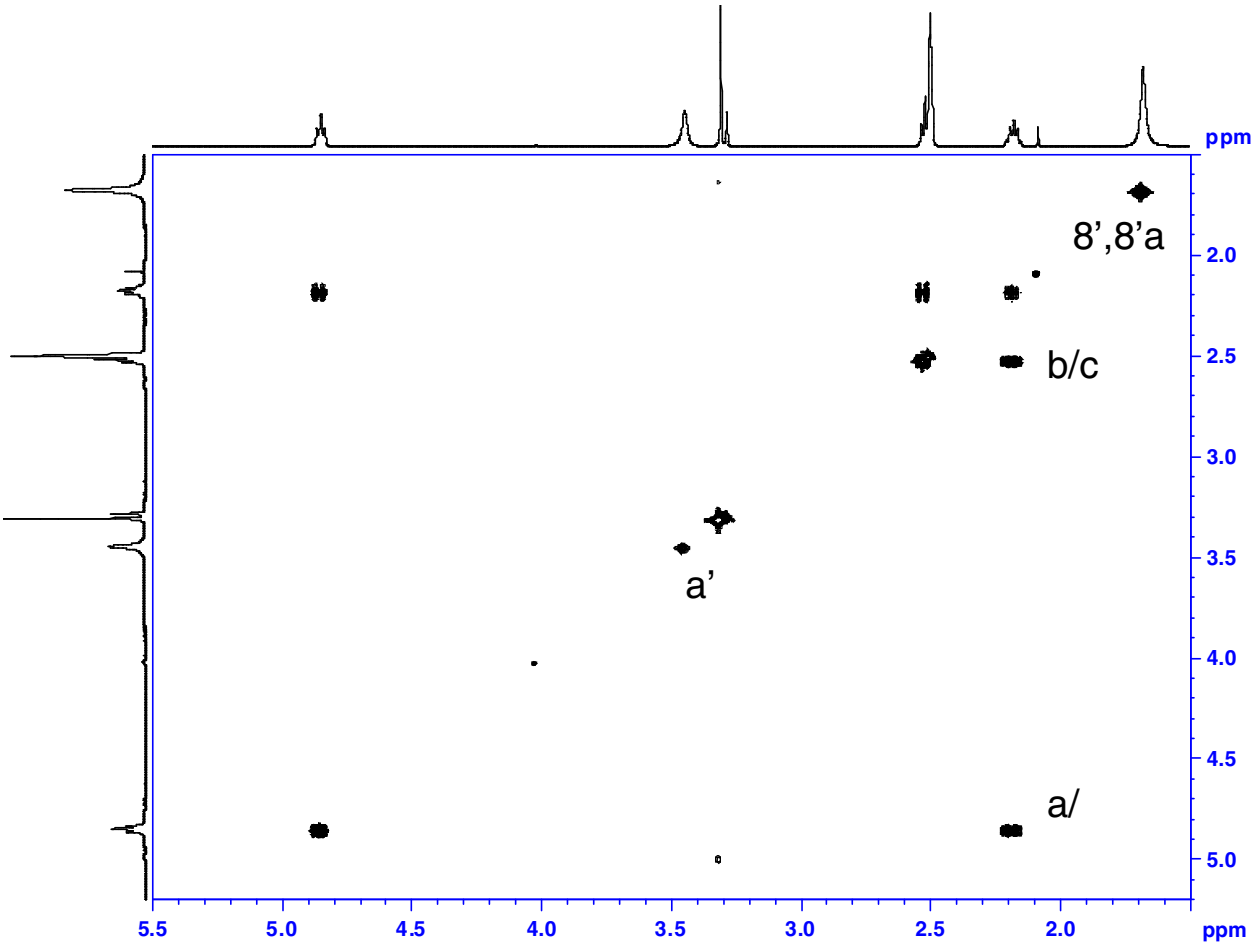


Spectrum 3: DIR ^{13}C NMR Expansion 2 (75.47 MHz, DMSO-*d6*)

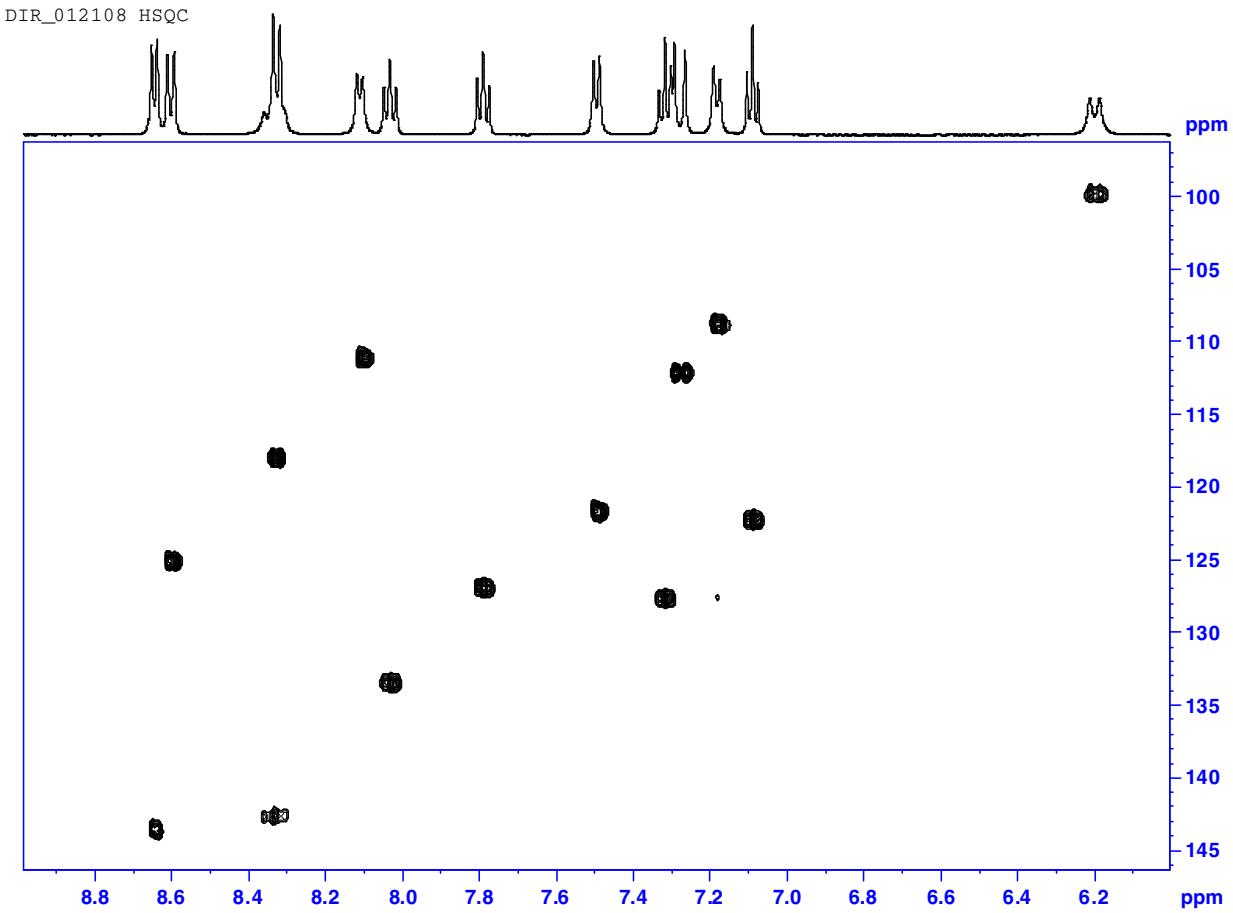
* quaternary carbons, assigned by HMBC experiments



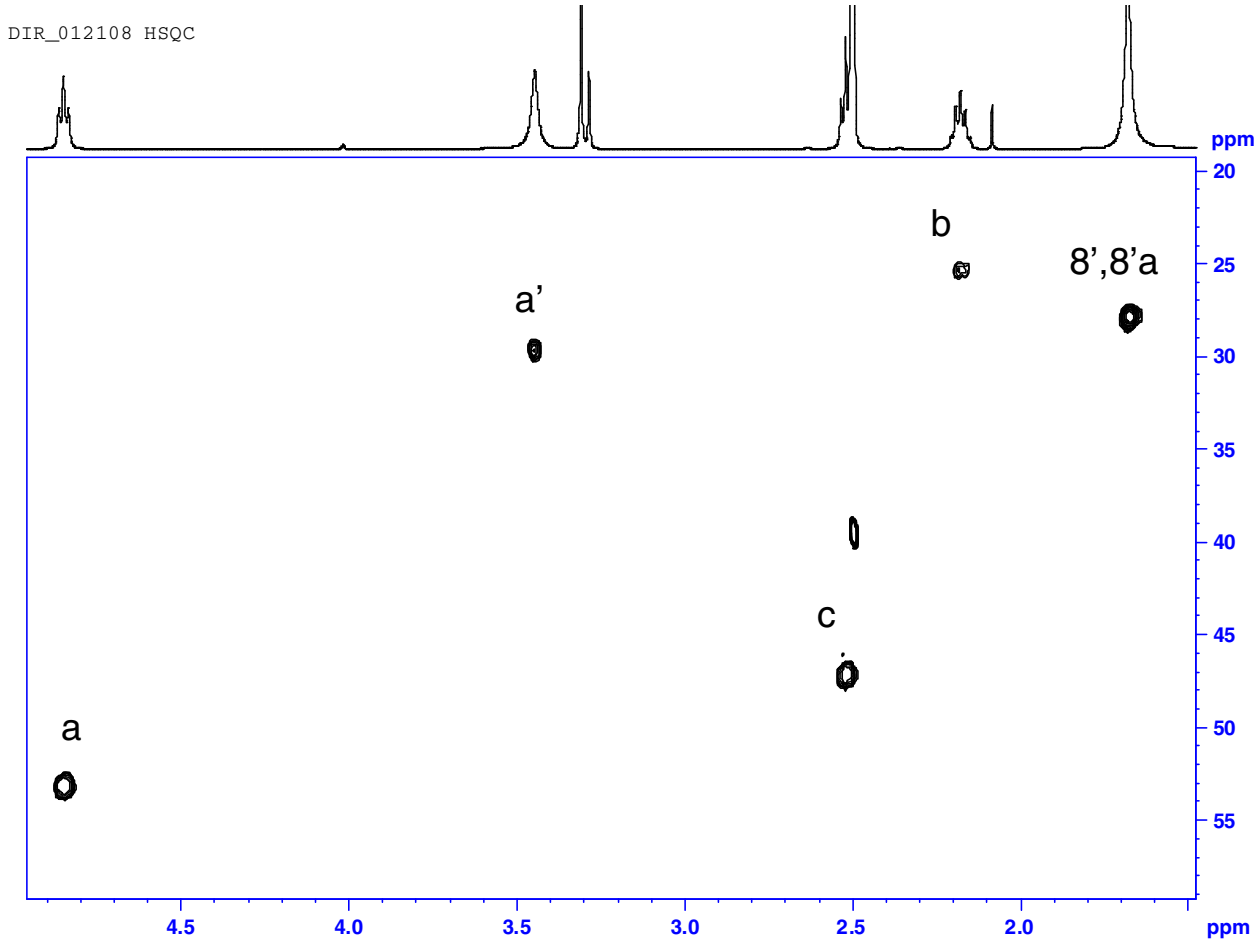
Spectrum 4: DIR: COSY Expansion 1



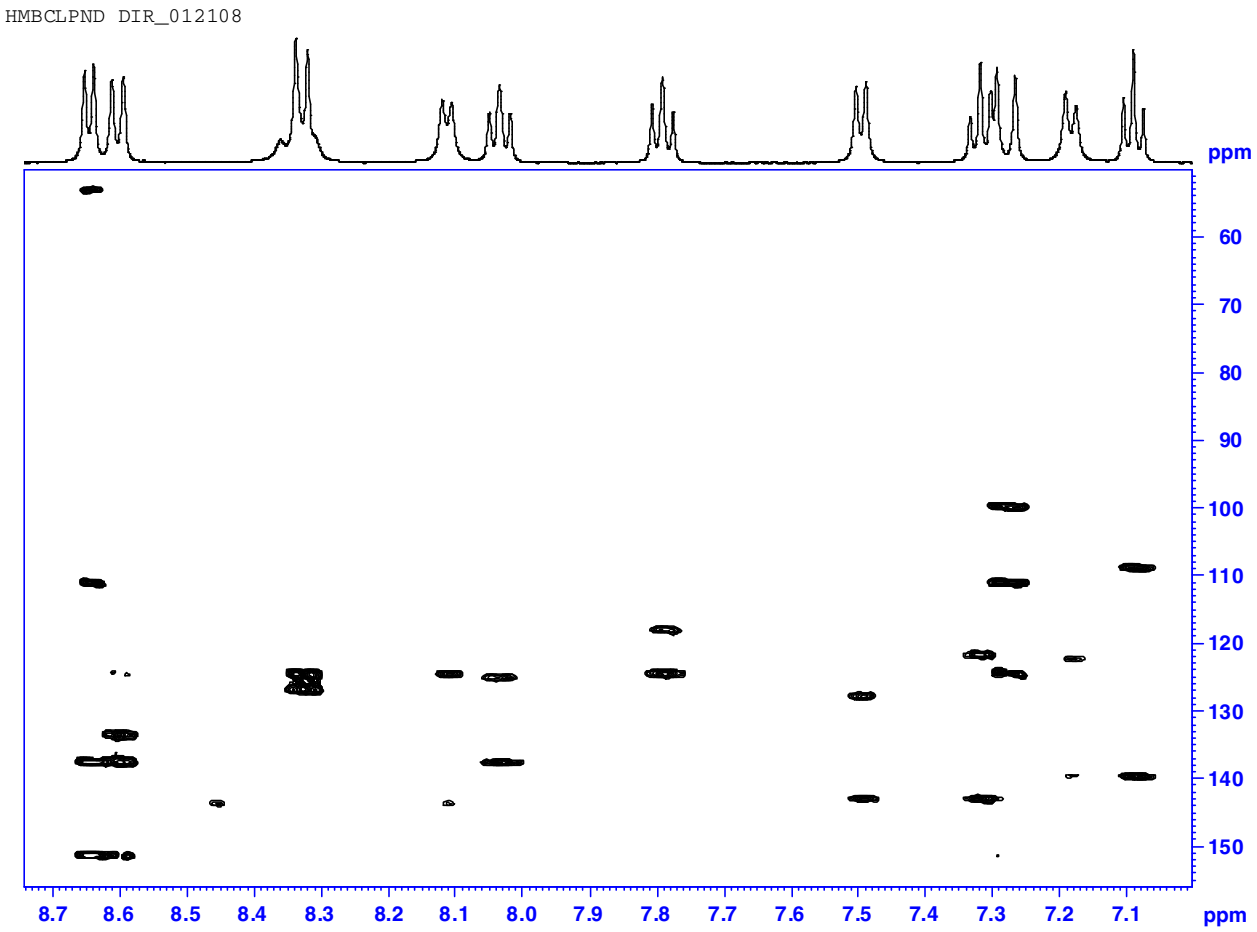
Spectrum 5: DIR COSY Expansion 2



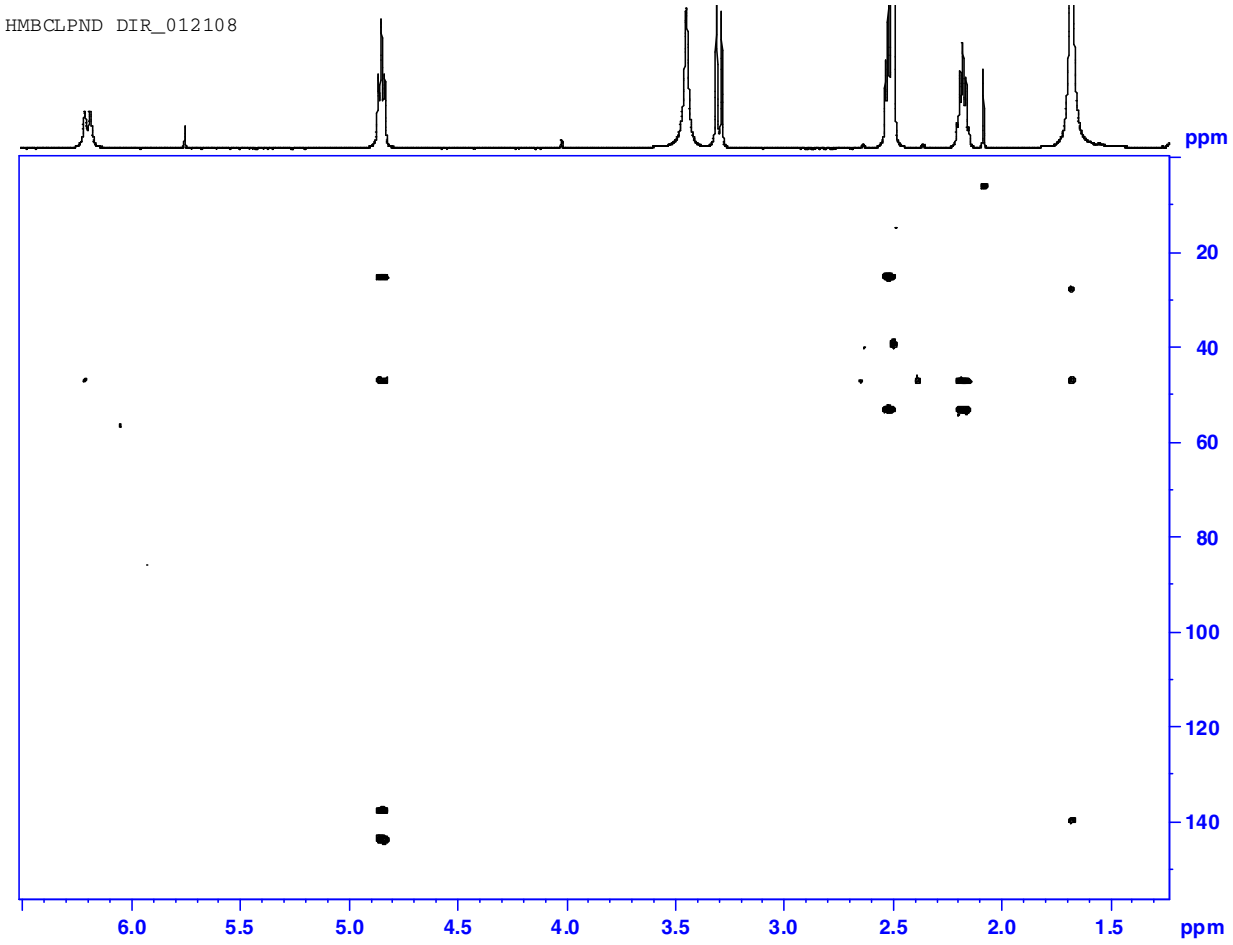
Spectrum 6: DIR HSQC Expansion 1



Spectrum 7: DIR HSQC Expansion 2



Spectrum 8: DIR HMBC Expansion 1



Spectrum 9: DIR HMBC Expansion 2