Supporting Information for

A cryptand based chemodosimetric probe for naked-eye detection of mercury(II) ion in aqueous medium and its application in live cell imaging

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SYNTHESIS

Rhodamine B hydrochloride (2 g, 4 mmol) was refluxed in 15 mL $POCl_3$ for 24 h and concentrated by evaporation under reduced pressure. The crude acid chloride was then dissolved in CH_3CN (25 mL) together with 2-bromoethylamine hydrochloride (2.0 g, 10 mmol) and triethylamine (2.5 mL) and stirred for 20 h at room temperature under N_2 atmosphere. The mixture was then concentrated under reduced pressure and organic part was extracted with chloroform (3 × 50 mL). The organic layer was then dried over Na_2SO_4 and concentrated by evaporation. The crude product was then purified by silica gel column chromatography using CH_3Cl/CH_3OH (99:1 v/v), affording **2** as pale pink solid. Yield ~30%; ^{1}H NMR spectra (500 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 1.16 (t, 12H, *J*=5.7 Hz), 2.97-3.00 (m, 1H), 3.12-3.15 (m, 1H), 3.29-3.37 (m, 8H), 3.43-3.46 (m, 1H), 3.49-3.53 (m, 1H), 6.25-6.27 (m, 2H), 6.37-6.43 (m, 4H), 7.05-7.07 (m, 1H), 7.41-7.45 (m, 2H), 7.89-7.90 (m, 1H); ^{13}C NMR spectra (125 MHz, $CDCl_3$, 25 °C, TMS): δ =

12.5, 28.7, 40.6, 41.7, 44.3, 64.7, 97.6, 104.9, 108.1, 122.9, 123.8, 128.1, 128.6, 130.5, 132.6, 148.8, 153.1, 153.5, 168.1; ESI-MS: m/z (%): 550.1792 (70) [M+2]$^+$; elemental analysis calcd (%) for $C_{30}H_{34}N_3O_2Br$: C 65.69, H 6.25, N 7.66; found: C 64.87, H 7.32, N 7.25.

Synthetic strategy for the fluorophore was based on the reactivity of the three -NH groups present in the three bridges of the cryptand[S1]. *Tris*-derivative of the cryptand can be achieved by simple aromatic nucleophilic substitution (ArSN) reaction where 3.5 equivalent amount of the reagent is allowed to react with the 1 equivalent of cryptand. The major *tris*-product can be easily separated by column chromatography.

To a solution of the cryptand, **L₀** (0.56 g; 1 mmol) in dry DMF (15 mL) was added anhydrous K_2CO_3 (0.44 g; 3.2 mmol). Subsequently N-bromoethyl Rhodamine B derivative (0.45 g; 3.5 mmol) was added gradually and the reaction mixture was allowed to stir at 110 ºC for 72 h. It was then poured into ice water (500 mL) after reducing the volume of DMF under reduced pressure. The brown solid separated was collected by filtration and washed thoroughly with water (5 × 100 mL). This yellow solid contains a mixture of *mono-*, *bis-* as well as *tris*-derivatives and was separated by column chromatography (SiO₂ 100-200 mesh). After removing the impurities and excess of reagents by CHCl₃, the *tris*-derivative was isolated using CHCl₃:MeOH (96:4 v/v) as the eluent. After evaporating the solvent, the brown solid was recrystallized from MeCN to obtain a crystalline solid of **CRD-1**. The synthetic route is given in Scheme 1.

CRD-1. Yield ~70%; ^{1}H NMR spectra (500 MHz, CDCl₃, 25 ºC, TMS): $\delta = 1.15$ (t, $J = 5.8$ Hz, 36H), 2.45 (t, $J = 4.4$ Hz, 6H), 2.64 (t, $J = 4.6$ Hz, 6H), 2.97-3.0 (m, 6H), 3.12-3.15 (m, 6H), 3.29-3.35 (m, 24H), 3.43-3.51 (m, 6H), 3.71 (s, 6H), 4.17 (t, $J = 4.4$

Hz, 6H), 6.25-6.27 (m, 6H), 6.37-6.42 (m, 12H), 6.82-6.89 (m, 6H), 7.05 (t, J = 1.4 Hz, 3H), 7.17-7.20 (m, 6H), 7.41-7.43 (m, 6H), 7.88-7.9 (m, 3H); ^{13}C NMR spectra (125 MHz, CDCl$_3$, 25 $^\circ$C, TMS): δ = 12.5, 28.6, 40.5, 41.7, 44.3, 47.3, 49.2, 54.8, 64.7, 67.5, 97.7, 105.0, 108.1, 111.2, 120.5, 122.8, 123.7, 128.0, 128.5, 128.6, 130.5, 131.0, 132.6, 148.8, 153.1, 153.5, 156.9, 168.1; ESI-MS: m/z (%): 1963.0439 (100) [M]$^+$; elemental analysis calcd (%) for C$_{123}$H$_{144}$N$_{14}$O$_9$: C 75.28, H 7.40, N 9.99; found: C 74.87, H 7.52, N 9.25.
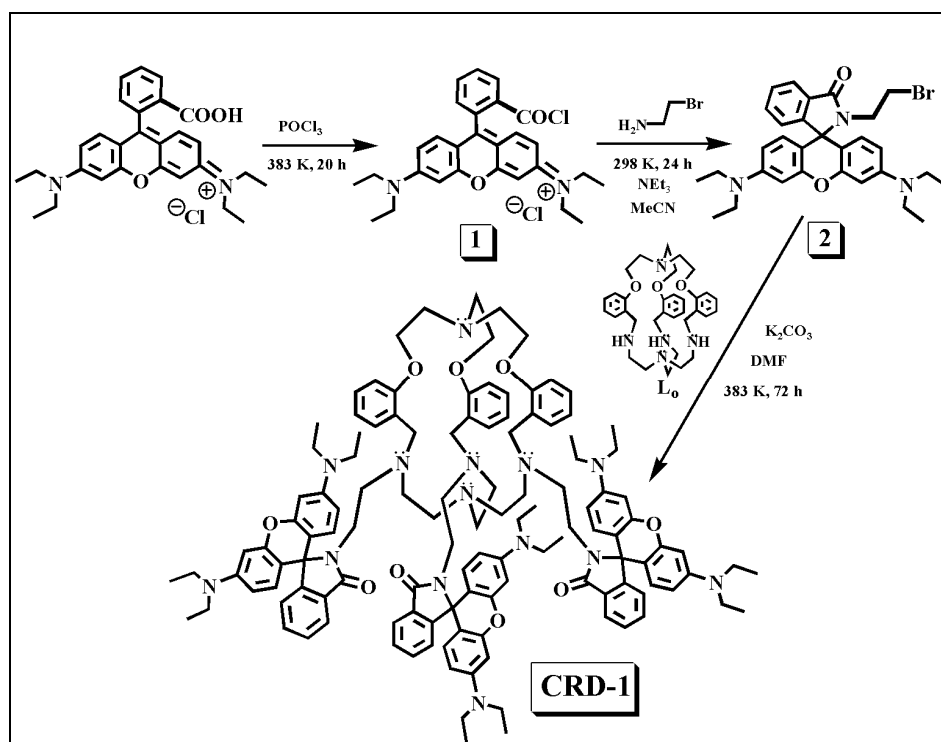
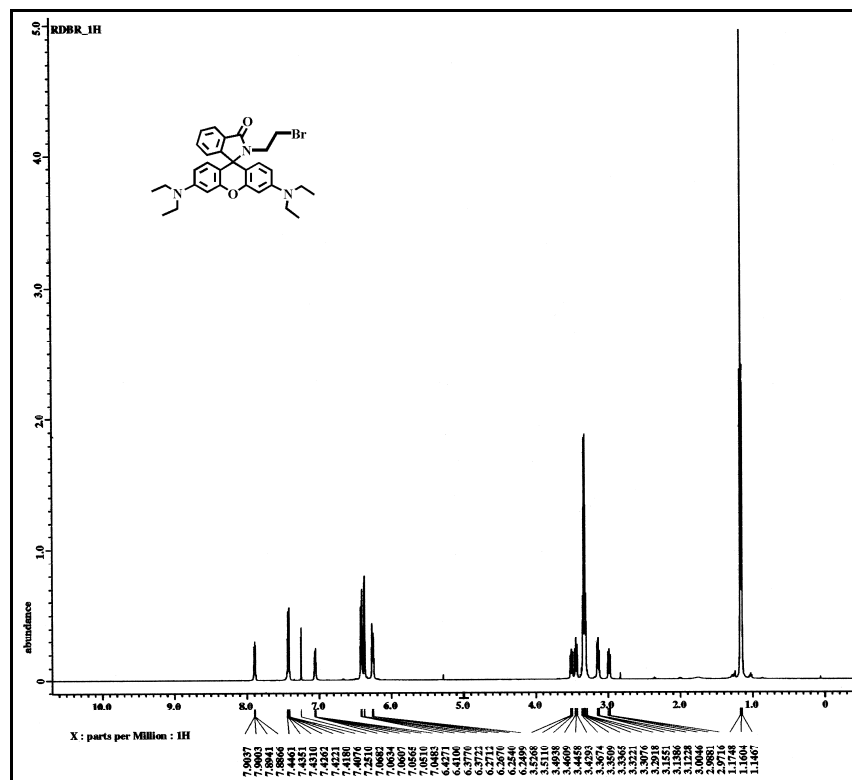**Scheme 1:** Synthetic scheme for the preparation of fluorophore **CRD-1** used in this study.

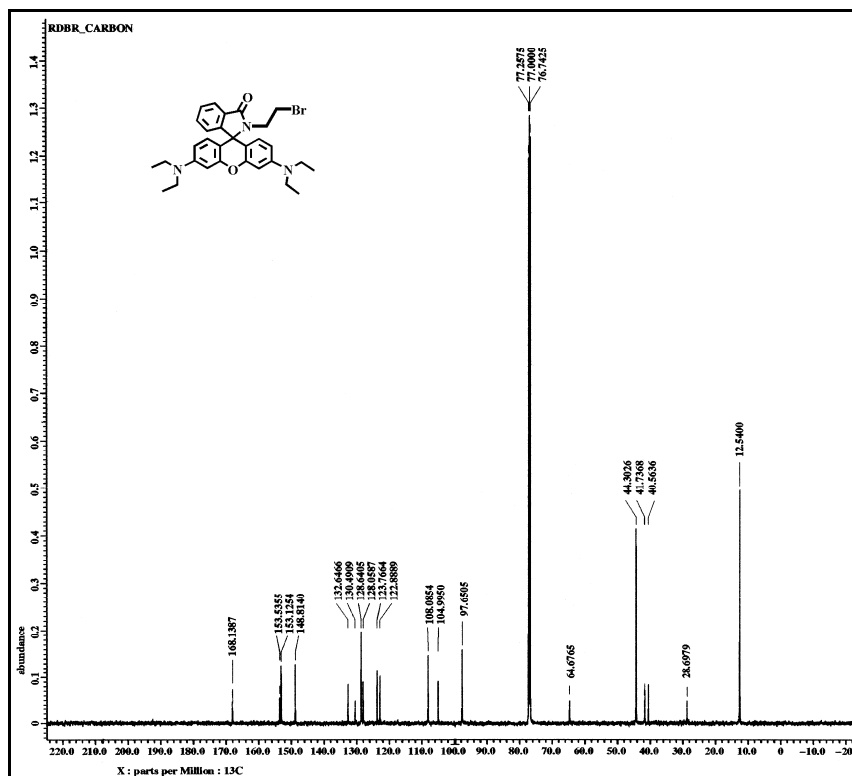*Figure S1*: 500 MHz ^{1}H NMR spectrum of **2** in CDCl$_3$

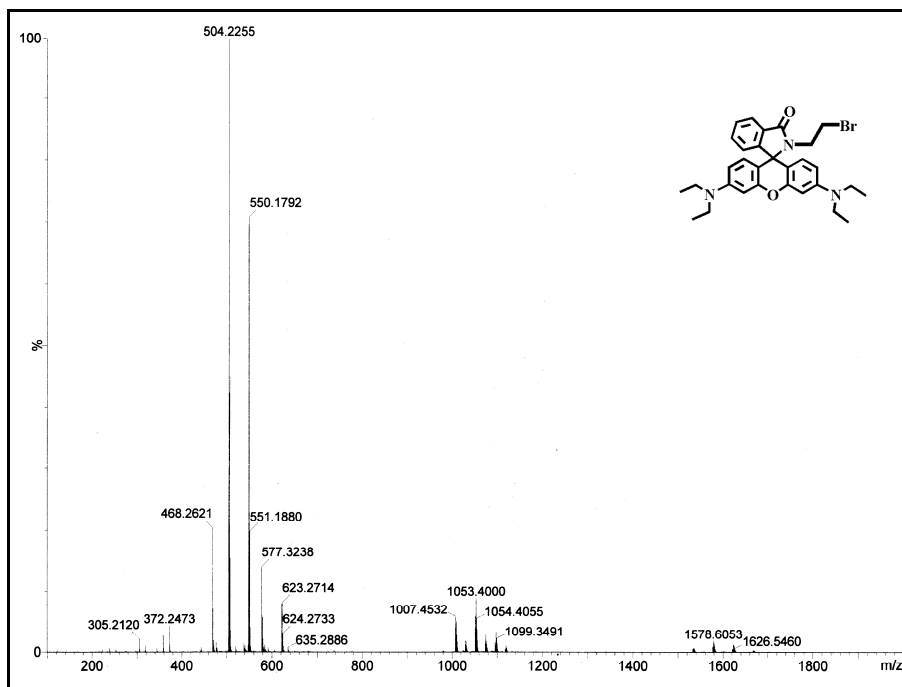*Figure S2*: 125 MHz ^{13}C NMR spectrum of **2** in CDCl$_3$
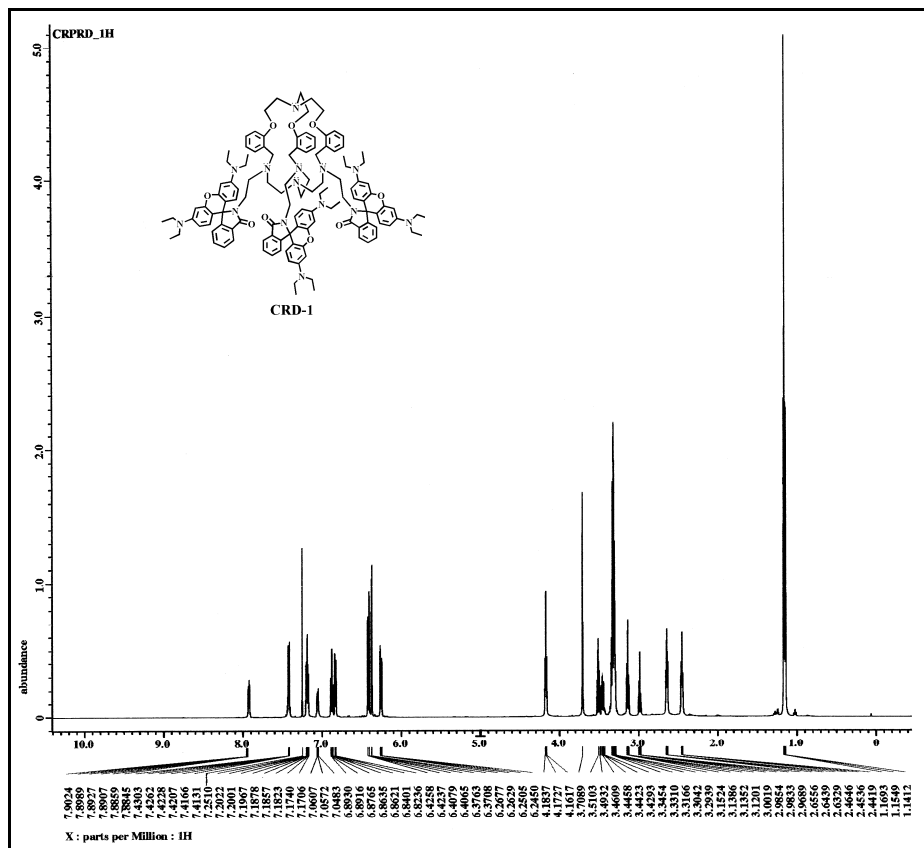
Figure S3: ESI MS of **2** in MeOH

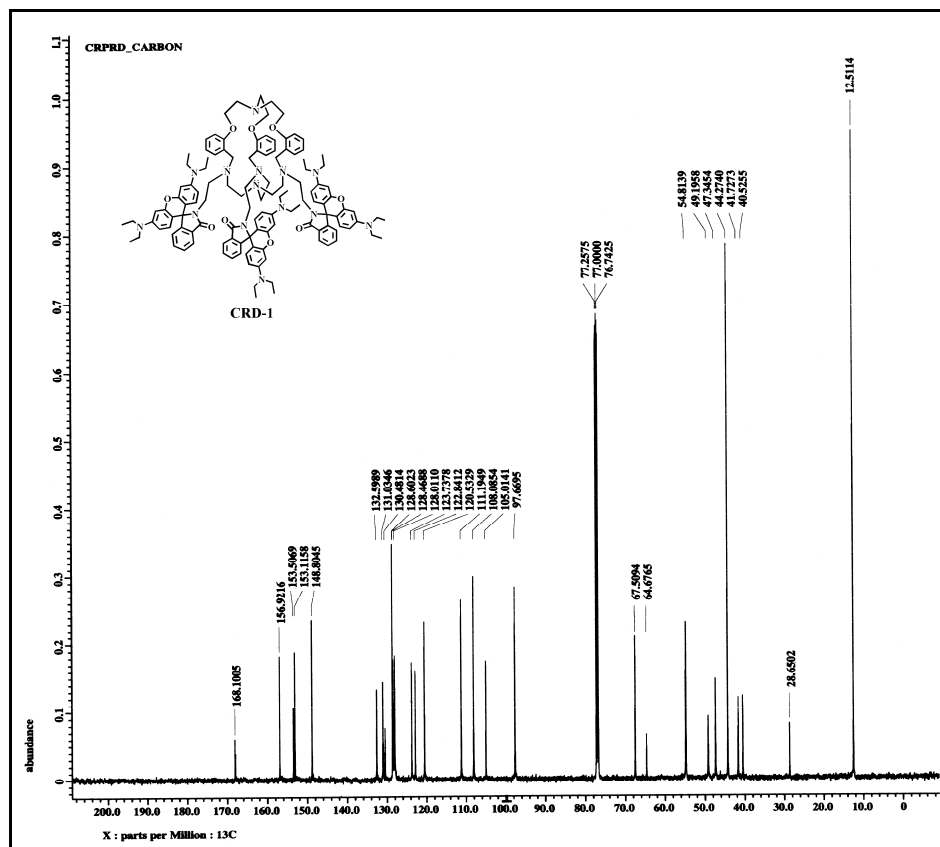*Figure S4:* 500 MHz ^{1}H NMR spectrum of **CRD-1** in CDCl$_3$

Figure S5: 125 MHz ^{13}C NMR spectrum of **CRD-1** in CDCl$_3$
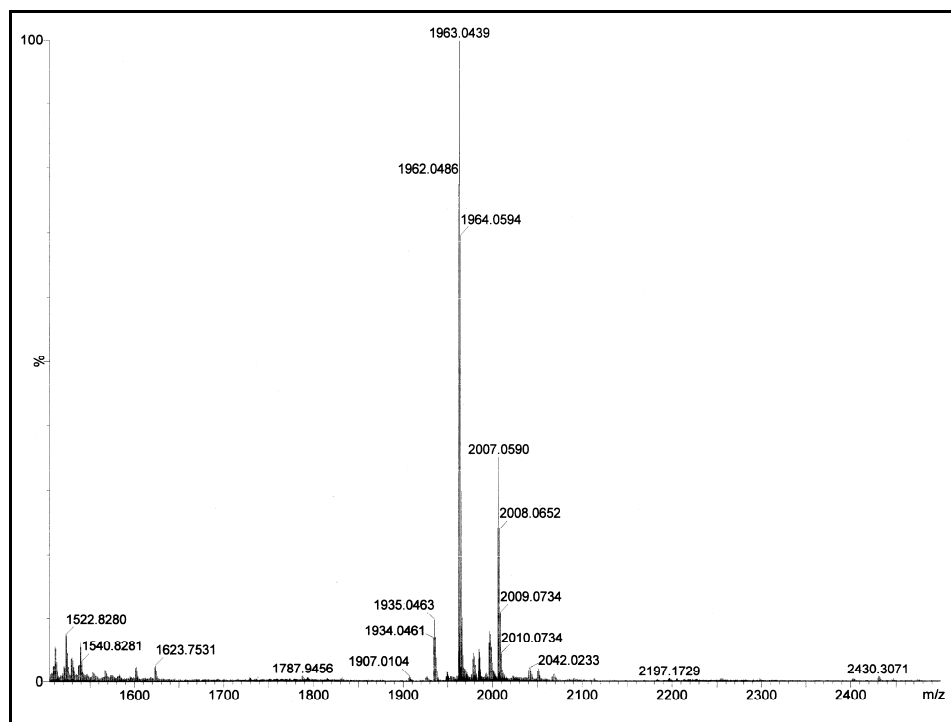

Figure S6: ESI MS of **CRD-1** in MeOH

Proposed Mechanism of Ring Opening Reaction for the Fluorophore

There are three secondary amines in the metal-free chromophore **CRD-1** that can participate in photo-induced electron transfer (PET) process. But in this case, like other rhodamine-based fluorophoric systems, a different type of mechanism is operational. Here, Hg(II) induced spirolactam bond-cleavage generates a fluorophore which is responsible for its observed emission behavior. The extent of spirolactam bond cleavage by different competitive metal ions determines their selectivity towards the dye, **CRD-1**.

The mechanism for Hg(II) induced ring opening reaction of the fluorophore is given in Scheme 2. A similar kind of ring opening reaction is available in the literature[S2], with crystallographic proof of the structure of the newly generated fluorophore after spirolactam bond cleavage. We believe the same type of mechanism is operating in our case. The ESI-MS also supports this mechanistic approach where it is clear that three Hg^{2+} ions are complexed with one dye molecule.
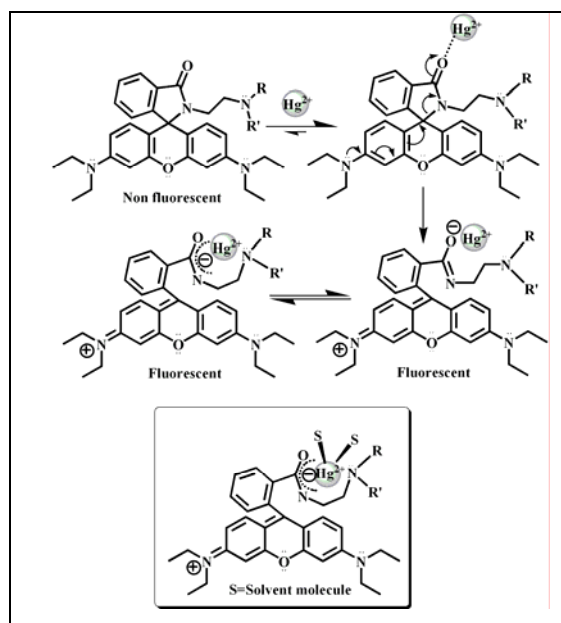

Scheme2. Proposed mechanism of spirolactam bond cleavage in the dye **CRD-1**.

Absorption Spectra

The UV-vis spectra for dye **CRD-1** were recorded in 20% water-ethanol mixture for all the metal ions including alkali, alkaline earth and transition metal ions at room temperature after keeping one day in dark. UV-vis titration also done in same condition, which clearly shows a 1:3 stoichiometry for **CRD-1**/Hg(II) complex with an association constant (K_s) of around 7×10^{11} M^{-3}. The stability constant (K_s) was determined using the following equation.

$$\log \frac{\left(A - A_{\min}\right)}{\left(A_{\max} - A\right)} = n \log c_M + \log K_s \ \ldots\ldots \ (\text{Eqn. 1})$$

Where, A is the absorption at the respective wavelength, $A_{\min}$ and $A_{\max}$ denote the values at the start and end point of the titration, n denotes the n:1 M/L stoichiometry and K_s is the overall stability constant.
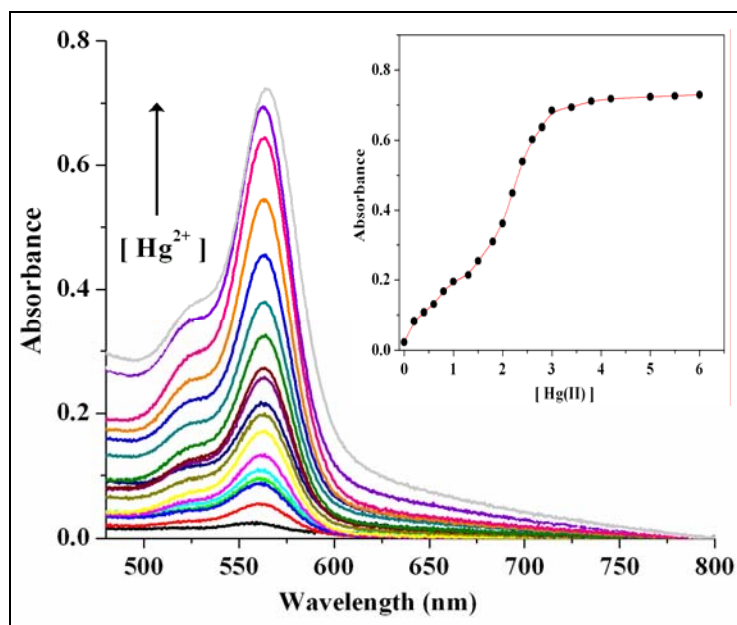
Figure S7: UV-vis titration results in 20% EtOH and H_2O mixture at pH = 7. The concentration of chromophore **CRD-1** is 10^{-4} M and the [Hg(II)] varies from 10^{-3} M to 10^{-5} M.

To confirm the stoichiometry between the dye **CRD-1** and Hg(II), electron spray ionization mass spectrometry (ESI-MS) also taken, which supports this experimental finding (Figure S8). The major peak (100%) at 468.2567 arises in ESI-MS is due to $[M+3Hg(II)+6MeCN]^{6+}$.
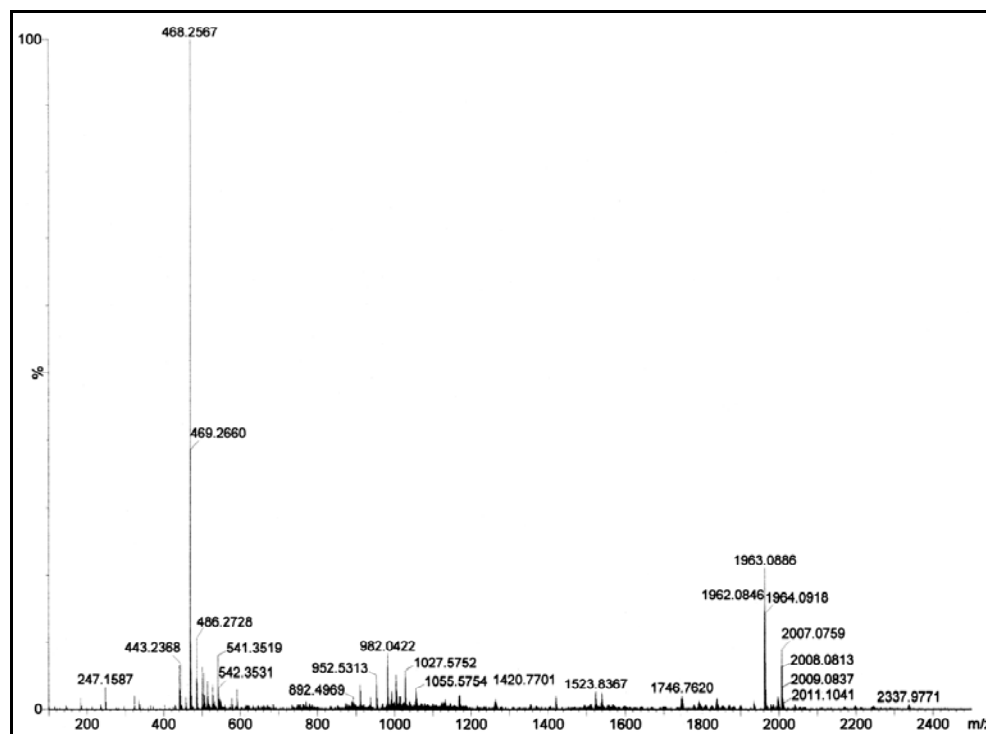
*Figure S8:* ESI MS of Hg^{2+} complex of **CRD-1** in MeCN

Selection of the Fluorophore

First of all our target is to find out the optimal condition for a good intracellular fluorescence imaging study. This generally concerns two important but fundamental requirements. (a) The dye must possess comparable fluorescence property in water (note that water is a fluorescence quencher) and (b) The dye must be amphiphilic in nature so that it can penetrate the cell wall quite easily.

Keeping these strategies in mind we have designed this dye molecule where the lipophilic cryptand was chosen as primary skeleton and hydrophilic rhodamine fragment

was grafted to this gradually. Then we have measured the emission behavior of all the three dye molecules where it is clear that the fluorescence enhancement occurs in the order of *mono- < bis- < tris-* derivative and we got maximum fluorescence output in case of *tris-* derivative which is given in Fig. S9. The fluorescence spectra were in 90% EtOH-H_2O mixture where all the three dye molecules have very good solubility. The excitation wavelength was 520 nm.

The major thing concerns its water solubility. The hydrophilic rhodamine makes the lipophilic cryptand core a proper amphiphillic dye after complete addition of three fragments. The *mono-* derivative is soluble in ~80-90% ethanol-water mixture. But for cellular imaging we can use a maximum of 1-2% ethanol or DMSO in water medium. Otherwise the living cell will dye. We get the satisfactory solubility test in case of *tris-* derivative only which was then selected for *in vivo* studies.
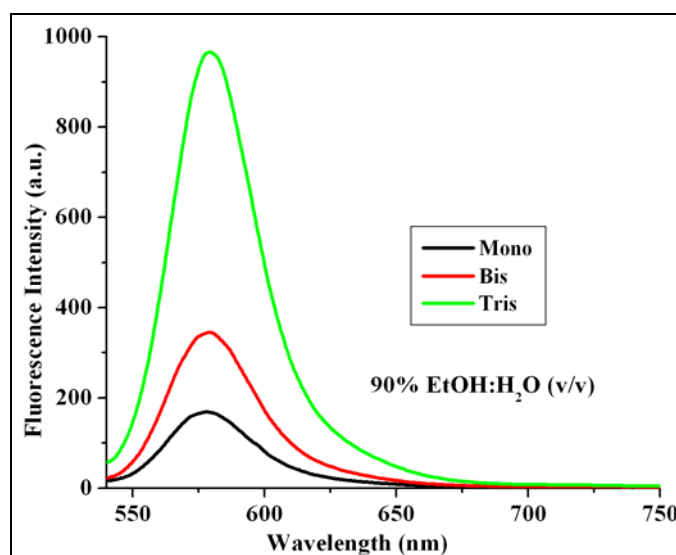
Figure S9. Fluorometric response of *mono-*, *bis-* and *tris-* rhodamine derivative of cryptand with 5 equiv of Hg^{2+} ion at ~90% EtOH-H_2O mixture. In each case the dye concentration was 1 μM. λ_{exc} = 520 nm.
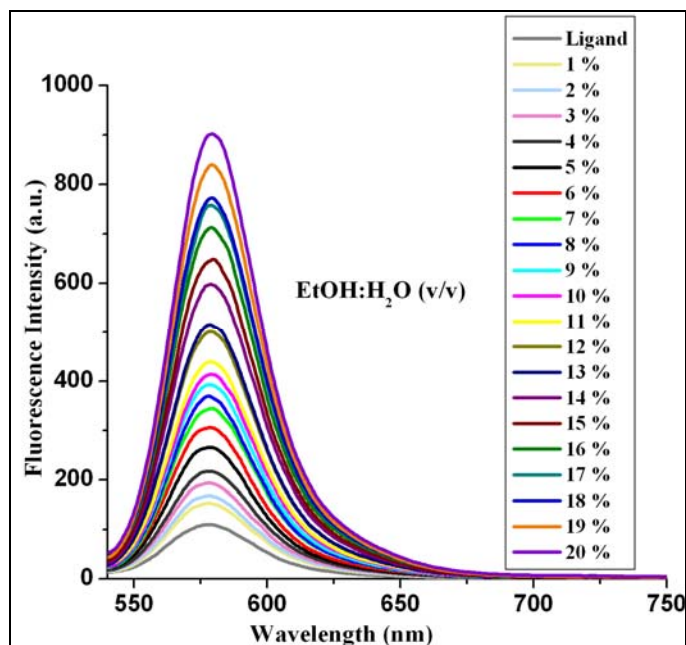
Figure S10. Fluorometric response of the dye with Hg^{2+} different ratio of ethanol and water at pH = 7; λ_{exc} = 520 nm. [**CRD-1**] is 10^{-6} M.
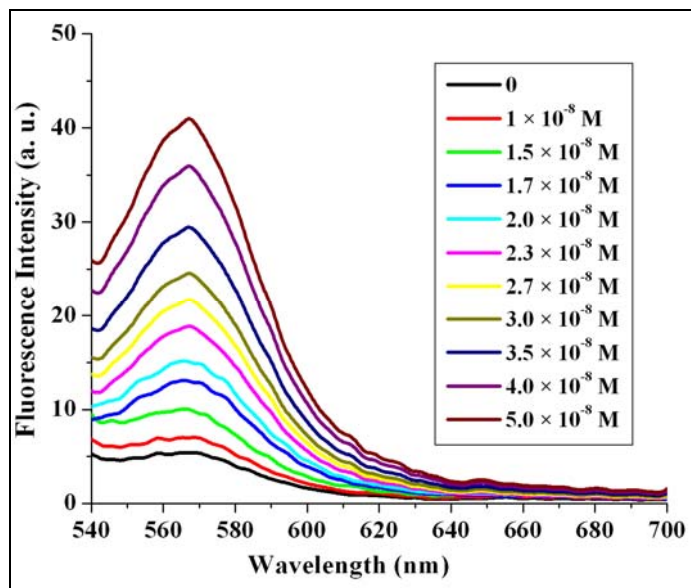
Figure S11: Change of fluorescence spectra of the dye **CRD-1** as a function of Hg(II) metal ion in ppb level in 1% EtOH–H$_2$O (v/v) mixture (λ_{exc} = 520 nm). [**CRD-1**] is 10^{-7} M and [Hg(II)] varies in the range of 1×10^{-8} M to 5×10^{-8} M.

Determination of Quantum Yield

The quantum yield was determined by the fluorometric method using the following equation.

$$\Phi_F = \Phi_{reference} \frac{(Area)_{sample}}{(O.D.)_{sample}} \left\{ \frac{(O.D.)_{reference}}{(Area)_{referenc}} \right\} \left\{ \frac{\eta^2_{sample}}{\eta^2_{reference}} \right\} \ldots\ldots \text{(Eqn. 2)}$$

Confocal fluorescence images with intracellular Hg^{2+} ion

HEK 293 cells (Human Embryonic Kidney cell lines) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal calf serum (HyClone), 100 μg/ml penicillin, 100 μg/ml streptomycin and 0.25 mM L-glutamine, at 37 °C in 5.0 % CO$_2$ humidified air for 7 days.

To determine the cell permeability of Hg^{2+}, all cell lines were incubated with **CRD-1** (1.0 μM) for 10–20 min at 37 °C, and washed with PBS to remove the remaining compound **CRD-1**. Upon addition of 1, 3 and 5 equiv of Hg^{2+} respectively, for 3 hours (at 1 h interval) to the HEK 293 cell line, the fluorescence image of intracellular Hg^{2+} was observed under a Zeiss LSM 510 META confocal microscope. Excitation wavelength of Argon laser was 514 nm and emission spectra were integrated over the range ~ 550 nm (single channel). For all images, the confocal microscope settings, such as transmission density, brightness, contrast, and scan speed were held constant to compare the relative intensity of intracellular Hg^{2+} fluorescence.
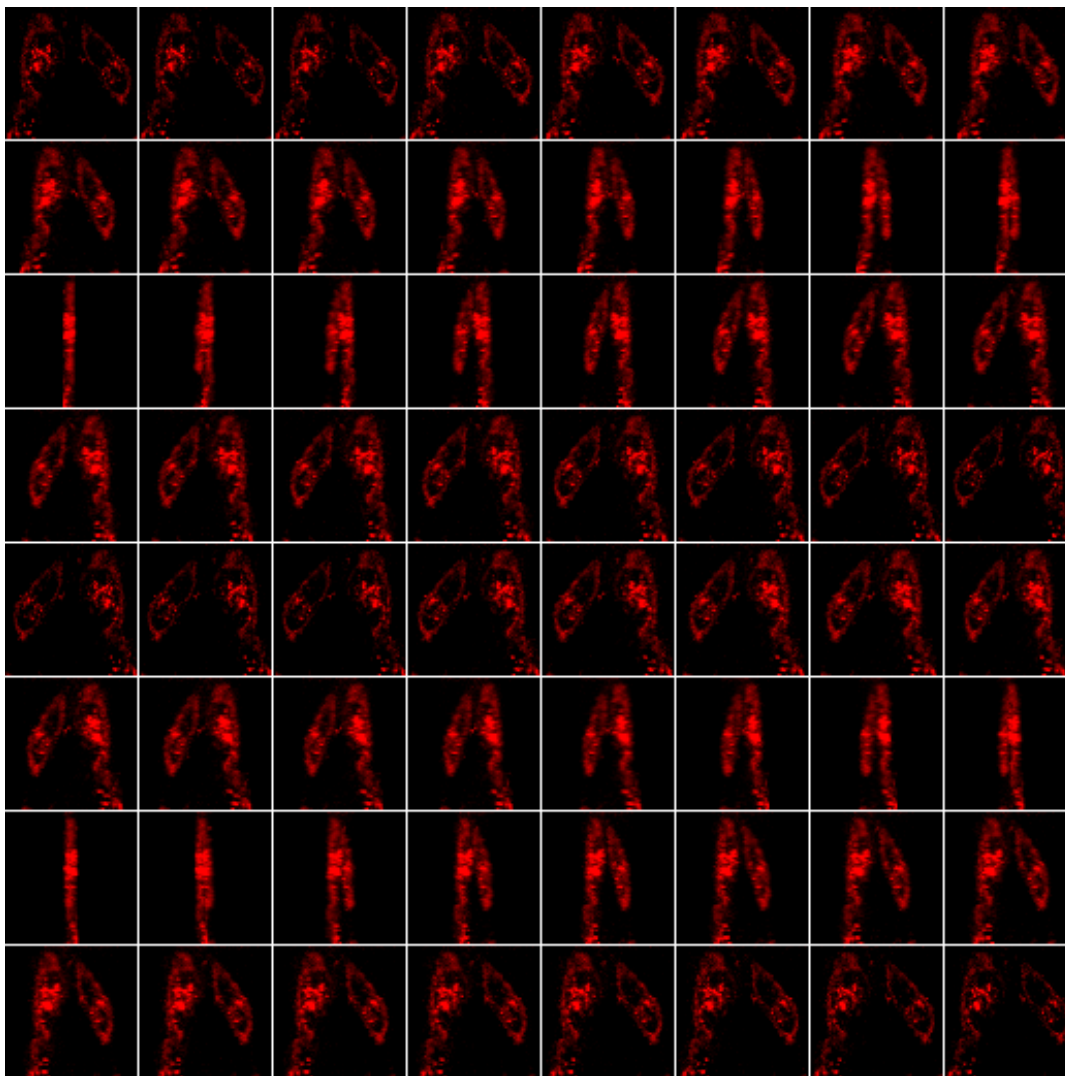
Figure 12. Confocal fluorescence 3D- slice images of HEK 293 cell incubated in **CRD-1** (1.0 µM) with 5.0 µM Hg^{2+} (Zeiss LSM 510 META confocal microscope, × 63 objective lens).

MTT assay

HEK 293 cell lines were inoculated into a 96-well, flat-bottomed microplate (Nunc, DNK) at a volume of 100 µl (5×104 cells/mL) for a stantionary culture. The media were exchanged into new fresh media. The cells were treated in the 2 fold down dilution series of **CRD-1** (5 µM) and then incubation in the 5% CO_2 at 37 °C, for 1 day. Cells were added MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

solution (2 mg/mL, Sigma). After an incubation in 5% CO_2 at 37 °C, for 4 hours, the solution was changed into 150 µl of dimethysulfoxide (DMSO:Kanto, JAP), shaked in microplate mixer (Amersham, UK) for 10 min. The optimal density (O.D.) value was measured by microplate reader (BIO-RAD 550, CA) using 540nm wavelength. The cell viability was calculated by the following formula. (Mean O.D. in treated wells ÷ Mean O.D. in control wells) × 100.
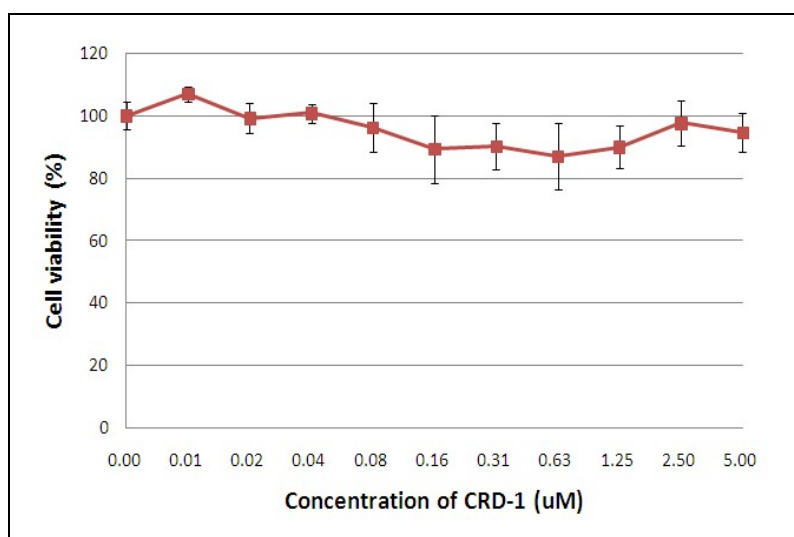
Figure 13. Percentage of HEK 293 cell viability remaining after cell treatment with **CRD-1**. (Untreated cells were considered to have 100% survival). Cell viability was assayed by the MTT method (values: mean ± standard deviation).[S3]

References

S1. K. G. Ragunathan and P. K. Bharadwaj, *Tetrahedron. Lett.*, 1992, **33**, 7581.

S2. D. Wu, W. Huang, C. Duan, Z. Lin and Q. Meng, *Inorg. Chem.*, 2007, **46**, 1538.

S3. For a detailed description of MTT assay, see: (a) Y. Cetin and L. B. Bullerman, *J. Agric. Food Chem.*, 2005, **53**, 6558; (b) I. Roy, T. Y. Ohulchanskyy, H. E. Pudavar, E. J. Bergey, A. R. Oseroff, J. Morgan, T. J. Dougherty and P. N. Prasad, *J. Am. Chem. Soc.*, 2003, **125**, 7860; (c) S. Andreescu, O. A. Sadik and D. W. McGee, *Chem. Res. Toxicol.*, 2005, **18**, 466.