DOI: 10.1002/ejic.201000512

Cell Compatible Fluorescent Chemosensor for Hg^{2+} with High Sensitivity and Selectivity Based on the Rhodamine Fluorophore

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Keywords: Rhodamine-B / Fluorescence / Mercury / Bioimaging / Sensors

An easily prepared "turn-on"-type fluorescent chemosensor for mercury based on Rhodamine-B, which exhibits high sensitivity and selectivity over other metal ions in aqueous systems, was obtained. The distinctive wavelengths in the UV/Vis absorption range can sense Hg^{2+}, Cu^{2+}, and Fe^{3+} separately. Furthermore, this "turn-on"-type fluorescent sensor, upon the addition of Hg^{2+} over other competitive species, was successfully applied to bioimaging in yeast and HeLa cells. The potential of these types of chemosensors for use in environmental and biological systems is great.

Introduction

As a toxic and hazardous metal, which distributes widely in air, water, and soil,[1] the mercury ion causes serious environmental and health problems because bacteria in the natural environment can convert inorganic mercury into neurotoxic methylmercury, which bioaccumulates through the food chain.[2] Furthermore, because of its high activity of producing toxic effects by binding strongly to sulfhydryl and to a lesser degree to hydroxy, carboxyl, and phosphoryl groups,[3] mercury can also easily accumulate in bodies of animals and cause numerous diseases such as pneumonitis, dermatitis, gastric disorders, anemia, and especially, neurological diseases.[4] Therefore, there is an urgent need for effective, rapid, and facile mercury ion detection, and so far much excellent work has been reported.[5] However, in order to make further progress, chemosensors with a less toxic action should have practical application in biological systems. Recently, several chemosensors have been successfully applied in living cells and tissues.[6]

Our strategy here is to utilize common molecules as a platform and to facilitate the reaction to obtain high sensitive, selective, and low toxic fluorescent probes, which could be applied in biological systems successfully. Herein, we synthesize compound **c**, (Scheme 1) in which Rhodamine B is connected to 2-hydroxy-1-naphthaldehyde through a hydrazine bridge; **c** is obtained through a one-step Schiff base reaction.[7] As a result of the excellent photophysical properties, such as high fluorescence, large absorption coefficient, long absorption and emission wavelengths,[8] and the well-known ring-opening mechanism from spirolactam derivatives (fluorescence "off") to its corresponding open-ring amide (fluorescence "on"),[9] a "turn-on"-type fluorescent and colorimetric probe (**c**) for Hg^{2+} with cell permeability and biocompatibility was obtained. Compound **c** does not only distinguish Hg^{2+}, Cu^{2+}, and Fe^{3+} by different UV/Vis absorption wavelengths, but also exhibits excellent selective and sensitive (2 ppb) fluorescent enhancement toward Hg^{2+}. Further, compared to most sulphur-bearing Hg^{2+} sensors,[5a,5c,5n,6a–6c] our compound may avoid the negative influence of sulfide toward natural systems.

Results and Discussion

In compound **c**, Rhodamine B acts as an electron acceptor, while β-naphthol acts as an electron donor through the hydrazine bridge (see Scheme 1). However, because of the existence of the spirolactam moiety, which inhibits internal charge transfer (ICT) between the xanthene moiety[10]and the lone pair of electrons on the nitrogen of –C=N– [resulting in a photoinduced electron transfer (PET)],[11] the compounds exhibit no absorption in the visible region.

The miscellaneous ions Hg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Na^{+}, Li^{+}, K^{+}, Ca^{2+}, Ag^{+}, Mg^{2+}, Cd^{2+}, and Ni^{2+} were used to evaluate the metal-ion bonding property and selectivity of this compound in methanol (Figure 1). Among these ions, only Hg^{2+} induces both a fluorescence enhancement and a red color change (Figure 2), while Cu^{2+} and Fe^{3+} exhibit a color change only.

Figure 1 shows the absorption of **c** in the presence of miscellaneous ions in methanol solution. The addition of Hg^{2+}, Cu^{2+}, Fe^{3+} obviously causes the appearance of new bands at 562, 551, and 555.5 nm, respectively. The absorption shift among the three ions is about 5 nm, which can be detected easily.

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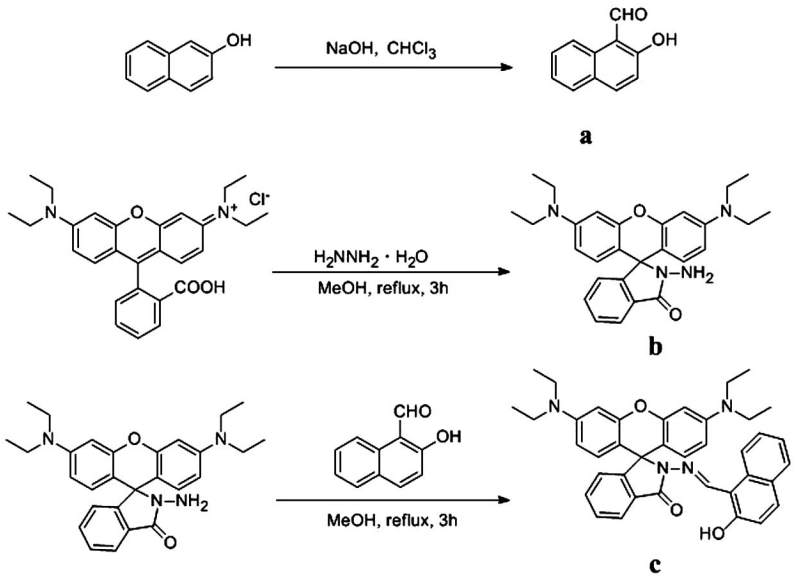
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejic.201000512.



Scheme 1. Synthesis of **c** and reference compounds.

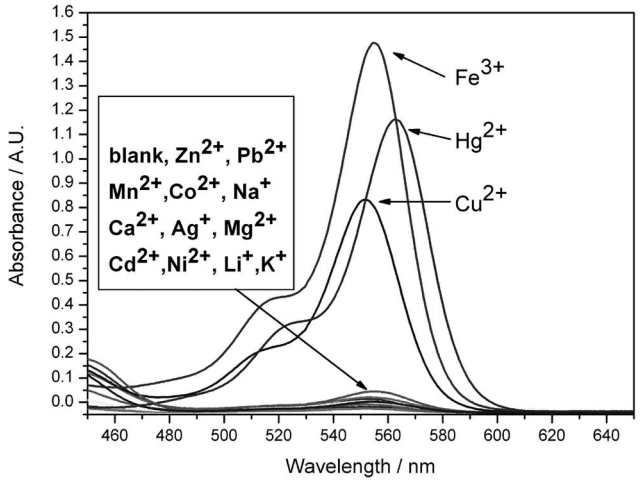


Figure 1. Absorption spectra of **c** (10 μM) upon addition of the miscellaneous ions (10 equiv.) in methanol solution at 25 °C.

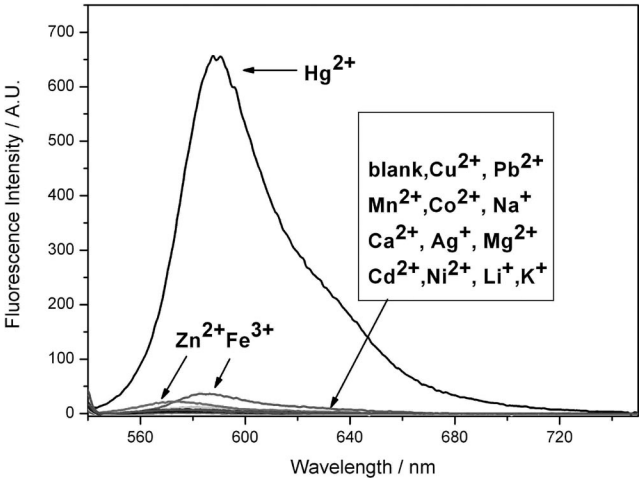


Figure 2. Fluorescence spectra of **c** (10 μM) in methanol with Hg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Na^{+}, Li^{+}, K^{+}, Ca^{2+}, Ag^{+}, Mg^{2+}, Cd^{2+}, Ni^{2+} (excitation at 535 nm) (excitation and emission slit 5 nm).

However, only Hg^{2+}, in spite of the color change caused by Cu^{2+} and Fe^{3+}, can trigger the fluorescence response of **c** (Figure 2) at 575 nm, while the other ions lead to much smaller or no fluorescence changes. The enhancement factor in the fluorescence of **c** is more than 300-fold upon the binding of Hg^{2+}. Figure 3 illustrates that the response of the fluorescence intensity of **c** toward Hg^{2+} is not influenced significantly by the addition of alkaline-earth metals and first-row transition metals. Both the UV/Vis and fluorescence results indicate that **c** shows great selectivity and sensitivity toward Hg^{2+} over other cations.

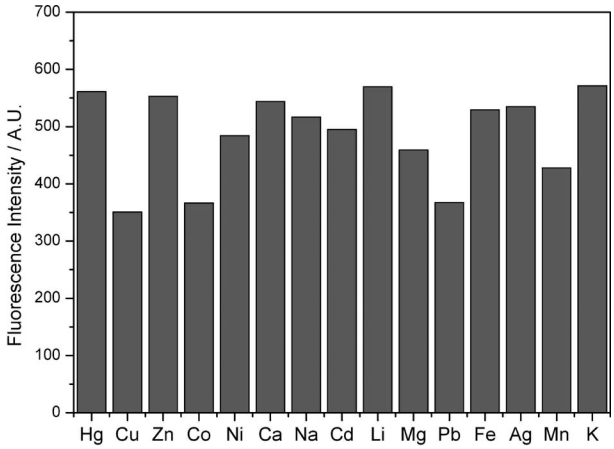


Figure 3. The fluorescence intensity change profiles of **c** in the presence of 1 equiv. Hg^{2+} and 2 equiv. of the interfering ions (Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Na^{+}, Cd^{2+}, Li^{+}, Mg^{2+}, Pb^{2+}, Fe^{3+}, Ag^{+}, Mn^{2+}, K^{+}, respectively).

Figure 4 shows the detailed absorption spectra of **c** in the presence of different concentrations of Hg^{2+} in methanol solution. The addition of increasing amounts of Hg^{2+} obviously causes the disappearance of the initial band at 371 nm attributed to the β-naphthol moiety and the appearance of a new band at 562 nm assigned to the ICT effect. The four well-defined isosbestic points at 313, 334, 380 and 460 nm

indicate the presence of a unique complex in equilibrium with the free ligand. Furthermore, a turning point at a 1:1 ratio of Hg^{2+}/ **c** can be observed in the plot of absorbance against the ratio of Hg^{2+} to **c**.

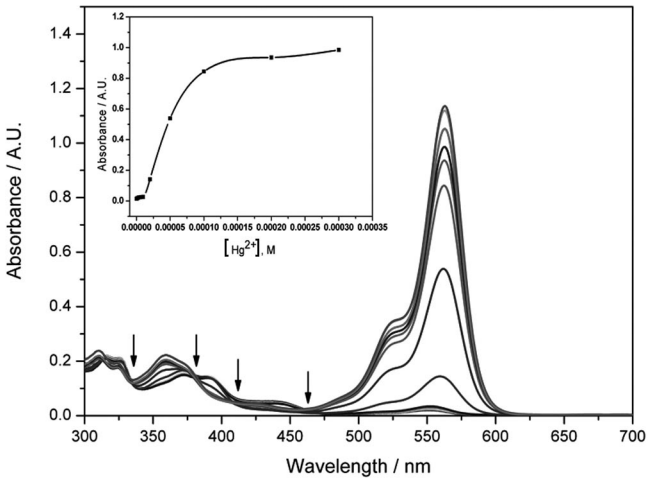


Figure 4. UV/Vis spectral changes of **c** upon the addition of increasing amounts of Hg^{2+} in methanol solution at 25 °C, [**c**] = 10 μM. The inset shows the plot of the absorbance at 562 nm in the presence of different concentrations of Hg^{2+}.

From the absorption titrations, the association constant of **c** with Hg^{2+} was found to be 3.9×10^{-5} (R = 0.9966) by using nonlinear least-square analysis shown in Figure S1.

The fluorescence titration of mercury was conducted with 10 μM **c** in methanol. Upon the addition of increasing concentrations of Hg^{2+}, a new emission band at 575 nm appears with increasing intensity. The detection limit is as low as 2 ppb (Figure 5).

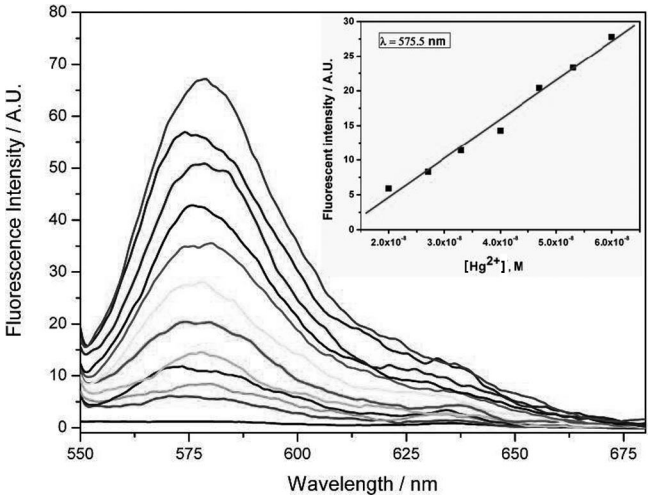


Figure 5. Fluorescence titration of **c** in the presence of 0–0.1 μM Hg^{2+}, [**c**] = 1 μM. The inset shows the detection limit of **c** to Hg^{2+}.

In the ESI-MS spectrum of **c** in the presence of Hg^{2+} (Figure S11), a peak at *m*/*z* = 843.17 (calculated value 843.28), which is assigned to [**c** + Hg^{2+} + CH_3OH–H^+]$^+$, is clearly observed upon addition of Hg^{2+}. This provides solid evidence for the formation of a 1:1 complex. Furthermore, this stoichiometry between **c** and Hg^{2+} is supported by the data obtained from the Job's plots of the absorption spectra (Figure S2).

To get an insight into the binding mode between **c** and Hg^{2+}, ^1H NMR and ^{13}C NMR spectra of complex **c**–Hg^{2+} and **c** in $CDCl_3$ were measured, as shown in Figure 6. The active protons are assigned as displayed in Figure 6a, where the downfield hydroxy hydrogen atom belongs to the naphthol moiety and the upfield hydrogen atom belongs to the xanthenyl moiety. All the peaks for the active protons broadened after the addition of Hg^{2+}, which indicates that proton exchange increases, and both the peak shifted upfield because of the electron-deshielding effect of Hg^{2+} on them. Moreover, when 1 equiv. Hg^{2+} was added, the peaks for the two protons disappeared, which implicates the carbonyl and hydroxy group of **c** in the bonding with Hg^{2+}.

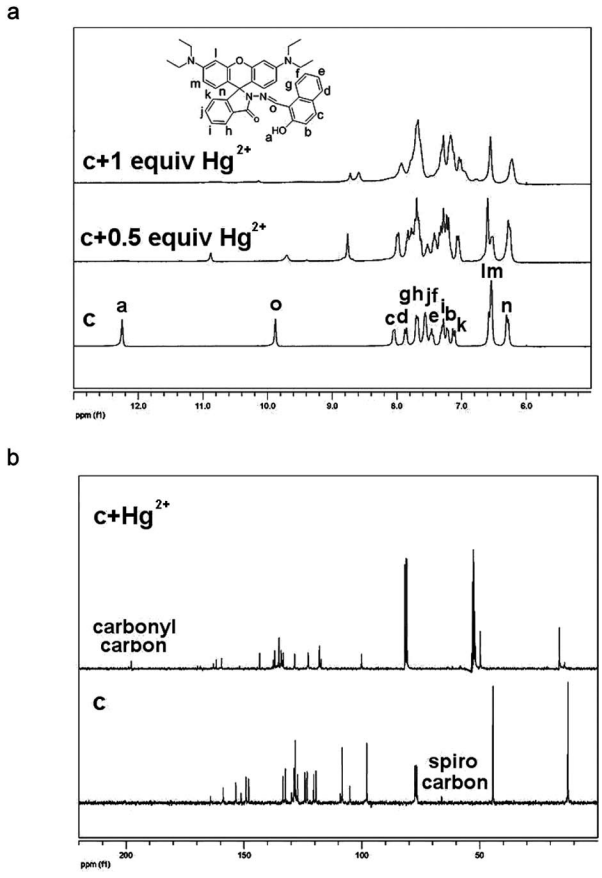
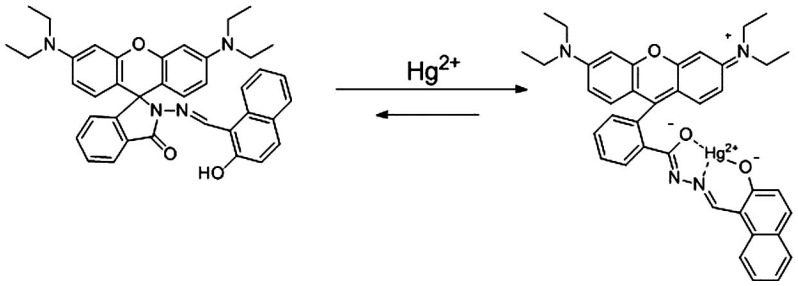


Figure 6. (a) Partial ^1H NMR (300 MHz) spectra of **c**, **c** + 0.5 equiv. Hg^{2+}, and **c** + 1 equiv. Hg^{2+}. (b) Partial ^{13}C MNR (75 MHz) spectra of **c** and **c** + 1 equiv. Hg^{2+}.

This fact is confirmed by ^{13}C NMR spectroscopy. The carbonyl carbon atom peak in the ^{13}C NMR spectra shifted from 164.2 to 197.8 ppm when 1 equiv. Hg^{2+} was added (Figure 6b). Moreover, the peak for the spiro carbon atom in **c** at δ = 66.1 ppm disappears and shifts to 143.3 ppm, which presents the formation of an open-ring form complex.



Scheme 2. Proposed mechanism for the fluorescent changes of **c** upon the addition of Hg^{2+}.

Accordingly, a reasonable explanation to these results that can be proposed is that Hg^{2+} induces the formation of an open spiro ring and is coordinated to a carbonyl oxygen atom, a naphthol oxygen atom, and an imino nitrogen atom, so that the xanthenyl moiety is restored in a manner similar to that in Rhodamine B and that the ICT effect is restored to show pink fluorescence (Scheme 2).

For practical applications, we needed to explore the influence of mercapto biomolecules in living cells, such as cysteine, which might interact with Hg^{2+}. As shown in Figure S3, the addition of 10 equiv. cysteine hardly brings about a change in fluorescence relative to that of the **c**–Hg^{2+} system. We then applied **c** to confocal fluorescence imaging of Hg^{2+} in yeast and HeLa cells (Figures 7 and 8, respectively). After staining the yeast cells with 40 μM **c** for 1 h, they display weak intracellular fluorescence (Figure 7b), and the intensity increases enormously after the further addition of 40 μM Hg^{2+} solution (Figure 7d). When **c** was added to the HeLa cells, they scarcely display any fluorescence (Figure 8b). After the addition of Hg^{2+} ions, the fluorescence intensity increases dramatically to show a clear red intracellular fluorescence (Figure 8d). The results indicate that **c** can permeate the cell membrane and combine with Hg^{2+} specifically, which would serve to indicate the presence of the free Hg^{2+} ion.

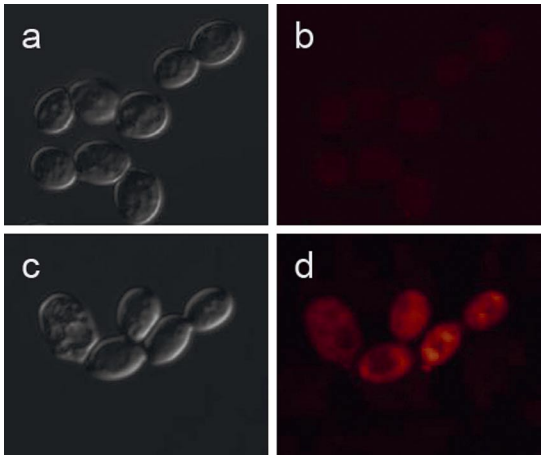


Figure 7. Confocal fluorescence and brightfield images of yeast cells: (a) and (c) Brightfield image of cells. (b) Cells incubated with a 40 μM solution of **c** for 1 h at room temperature. (d) Cells treated with a 40 μM solution of **c** for 1 h and then incubated with 40 μM Hg^{2+} for 30 min at room temperature. (λ_{ex} = 546 nm, λ_{em} = 560–600 nm, 1200×).

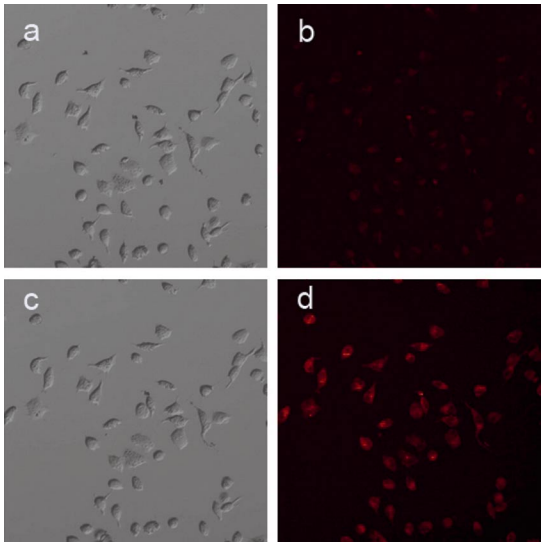


Figure 8. Confocal fluorescence and brightfield images of HeLa cells: (a) and (c) Brightfield image of cells. (b) Cells incubated with a 10 μM solution of **c** for 10 min at room temperature. (d) Cells treated with a 10 μM solution of **c** for 10 min and then incubated with 10 μM Hg^{2+} for 10 min at room temperature. (λ_{ex} = 546 nm, λ_{em} = 560–600 nm, 300×).

The bioimaging tests on the yeast and HeLa cells confirm that **c** provides a fluorescence enhancement with excellent cell-permeability and biocompatibility for tracing the Hg^{2+} ion in cells, specifically and rapidly. As a result, **c** could be considered as an effective Hg^{2+}-specific fluorescence sensor for the detection of the Hg^{2+} uptake in organisms.

Conclusions

We have designed and synthesized a new colorimetric fluorescent chemosensor **c** based on rhodamine B for the mercury ion. It exhibits extremely high sensitivity (as low as 2 ppb) and selectivity to Hg^{2+} over other cations, especially Cu^{2+} and Fe^{3+} and leads to a great fluorescence enhancement Further characterization confirms a 1:1 complex, which restores the ICT effect to show fluorescence. The ex-

cellent biological value of **c** is demonstrated by the fluorescence imaging in living yeast and HeLa cells.

Experimental Section

General: Methanol was of HPLC grade and obtained from Merck. All other reagents were of analytical grade unless noted. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Ultrashield 300 MHz NMR spectrometer. UV/Vis and fluorescence spectra were recorded on a Varian Cary 50 Probe UV/Vis spectrophotometer. Mass spectroscopy was recorded with a Thermo LCQ Fleet MS spectrometer. Yeast (Saccharomyces Cerevisiae) and HeLa cell lines were provided by the School of Life Science, Nanjing University. The biological imaging tests were carried out with an Olympus FV-1000 laser scanning confocal fluorescence microscope.

Compound a: β-Naphthol (50 g) and ethanol (175 mL) was added into a three-neck flask and stirred vigorously. A solution of NaOH (100 g) and water (200 mL) was then added quickly. Chloroform (146 mL) was added drop by drop when the mixture was heated to 80 °C. Stirring was maintained for 1 h after the addition of chloroform was complete, and sodium 1-formlnaphthalen-2-olate then precipitated. Ethanol and chloroform were removed under reduced pressure. Hydrochloric acid was then added to the residual solutions until the pH of the solution was 4–6. The solution was extracted by chloroform and dried by sodium sulfate. After removal of chloroform, the resulting solid was distilled under reduced pressure. Yield: 17.5 g (30%), white needlelike solid **a** (2-hydroxy-1-naphthaldehyde). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.18 (d, $^3J_{H,H}$ = 6.00 Hz, 1 H, naphthalene), 7.43–7.47 (m, 1 H, naphthalene), 7.59–7.65 (m, 1 H, naphthalene), 7.79–7.82 (m, 1 H, naphthalene), 8.03 (d, $^3J_{H,H}$ = 9.00 Hz, 1 H, naphthalene), 8.33 (d, $^3J_{H,H}$ = 8.70 Hz, 1 H, naphthalene), 10.81 (s, 1 H, CHO), 13.16 (s, 1 H, OH) ppm. IR (KBr): $\tilde{\nu}_{max}$= 3480, 1701, 1630, 1315, 1161, 745 cm^{-1}.

Compound b: To rhodamine B (4.98 g, 10.4 mmol) in methanol solution (8 mL) in a 50-mL round-bottomed flask was added hydrazine hydrate (3 mL). The solution was then heated at reflux and stirred for 3 h. After cooling to room temperature, a red solid precipitated and was washed by cool methanol until the filtrate turned colorless. Yield: 73%, red powder **b** (2-amino-3′,6′-bis(diethylamino)spiro[isoindoline-1,9′-xanthen]-3-one), kept without light. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.95 (m, 1 H, benzene), 7.47 (t, $^3J_{H,H}$ = 3.9 Hz, 2 H, benzene), 7.10–7.13 (m, 1 H, benzene), 6.43–6.49 (m, 4 H, benzene), 6.31 (dd, $^3J_{H,H}$ = 2.4, $^3J_{H,H}$ = 9.0 Hz, 2 H, benzene), 3.36 (q, $^3J_{H,H}$ = 6.9 Hz, 8 H, methylene), 1.18 (t, $^3J_{H,H}$ = 6.9 Hz, 12 H, methyl) ppm.

Compound c: To compound **b** (0.7 g, 1.5 mmol) in methanol solution (16 mL) in a 50-mL round-bottomed flask was added **a** (1 equiv.). The solution was then heated at reflux for 2.5 h. After cooling to room temperature, a straw-yellow solid precipitated. The solid was washed by cool methanol until the filtrate turned colorless and was dried in vacuo. Yield: 0.75 g (80%), straw-yellow powder **c**. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 12.25 (s, 1 H, C-OH), 9.87 (s, 1 H, benzylidenimin), 8.04–8.05 (m, 1 H, naphthalene), 7.84 (d, $^3J_{H,H}$ = 7.5 Hz, 1 H, naphthalene), 7.68 (d, $^3J_{H,H}$ = 5.1 Hz, 2 H, naphthalene), 7.56 (s, 2 H, naphthalene), 7.46 (s, 1 H, benzene), 7.21 (d, $^3J_{H,H}$ = 3.9 Hz,1 H, benzene), 7.10 (t, $^3J_{H,H}$ = 4.2 Hz, 1 H, benzene), 6.54 (s, 4 H, benzene), 6.28 (d, $^3J_{H,H}$ = 7.2 Hz, 3 H, benzene), 3.32 (q, $^3J_{H,H}$ = 6 Hz, 8 H, methylene), 1.13 (t, $^3J_{H,H}$ = 6.9 Hz, 12 H, methyl) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 164.2, 158.8, 153.5, 151.2, 149.2, 148.0, 133.5, 132.5, 129.8, 128.7, 128.6, 128.3, 128.0, 127.2, 124.1, 123.3, 123.2, 120.4, 119.4, 109.1, 108.3, 105.1, 97.1, 77.5, 77.1, 76.6, 66.2, 44.3, 12.6 ppm. IR (KBr): $\tilde{\nu}_{max}$= 3056, 2975, 1720, 1616, 1518, 1311, 1125, 822, 787, 750 cm^{-1}. ESI-MS (negative ion mode): = *m*/*z* = 609.42 $[M - H]^-$.

Cell Imaging

Yeast Cell Imaging: *Saccharomyces Cerevisiae* was cultured in the YPD liquid medium (peptone 20 g, yeast extract 10 g, dextrose 20 g, distilled water 1000 mL) for 12 h at 30 °C. For cell staining, the cells were incubated with a 40 μM solution of **c** in Tris-HCl (0.01 M, pH 7.2) for 1 h at 30 °C. The sensor solution was then removed, and the cells were washed twice with PBS (0.01 M, pH 7.4) to remove extracellular **c**. The cells were subsequently divided into two groups. The first is a control group without the addition of Hg^{2+} solution; the other was treated with a 40 μM solution of Hg^{2+} for 30 min at 30 °C. The cells were dropped on glass slides and excited at 546 nm by using a He–Ne laser. The emission was monitored from 560 to 600 nm.

HeLa Cell Imaging: HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C and 5% CO_2. Before staining, the cells were washed twice with fresh DMEM, and subsequently exposed to a 10 μM solution of **c** (900 μL DMEM added with 100 μL of a 100 μM solution of **c** in DMSO) for 10 min at room temperature. After washing twice with fresh DMEM, the cells were immersed for 10 min into a 10 μM solution of Hg^{2+} (900 μL DMEM added with 100 μL of a 100 μM solution of $HgCl_2$ in H_2O), DMEM was then removed, and the cells were washed twice with fresh DMEM and imaged. Excitation was at 546 nm, and emission was monitored from 560 to 600 nm.

Supporting Information (see footnote on the first page of this article): Fluorescence and absorbance spectra, NMR spectroscopic data and ESI-MS data are presented.

Acknowledgments

This work was supported at Nanjing University by the Science Foundation of Jiangsu Province (BK2006717) and the National Natural Science Foundation of China (40930742).

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Received: May 10, 2010
Published Online: August 4, 2010

