ORGANIC LETTERS

2012
Vol. 14, No. 1
406–409

# Highly Selective Fluorescence Turn-on Chemodosimeter Based on Rhodamine for Nanomolar Detection of Copper Ions

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**Received November 29, 2011**

## ABSTRACT


**A highly selective fluorescent chemodosimeter based on rhodamine is synthesized which undergoes $Cu^{2+}$ driven hydrolysis in aqueous media to produce fluorescence turn-on changes with a detection limit up to the nanomolar range.**

Copper is an essential soft transition metal ion that plays a crucial role in environmental, biological, and chemical systems. Copper, being both useful and cytotoxic, is a vital trace element for the activities of enzymes because of its redox-active nature.[1] Copper deficiency may lead to hematological manifestations[2] and a wide variety of neurological problems.[3] An excess amount of copper in the body causes gastrointestinal disturbance and damage to the liver and kidneys.[4] Thus, in view of the significance of copper ions, simple and rapid sensing of copper ions in biological[5] and environmental systems is very important. Copper ions, being of a catalytic nature, promoted the hydrolysis of amides and esters, reactions including oxidations, dethioacetalizations, rearrangements, and oxidative cyclizations.[6] The method of copper induced reactions has been an excellent approach for the development of selective chemodosimeters for these ions. For example, Han and coworkers[6e] and Li et al.[6k] reported efficient fluorescent chemodosimeters which catalytically hydrolyzed in the presence of $Cu^{2+}$ ions. However, the reported examples of $Cu^{2+}$ mediated hydrolysis to induce fluorescence enhancement generally involve the generation of a single fluorescent species and required a prolonged reaction time to give the desired fluorescence change. The development of chemodosimeters which undergo $Cu^{2+}$ mediated hydrolysis to generate more than one fluorescent species is still a

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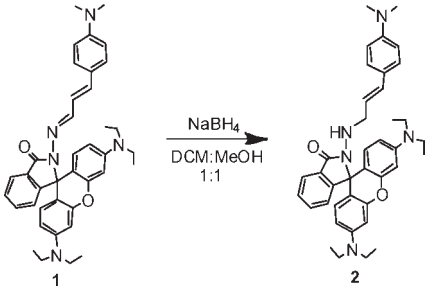
10.1021/ol203186b 
**Published on Web 12/15/2011**

challenge, yet this may open a new approach for the construction of fluorescent chemodosimeters for desired analytical objectives.

Our research work involves the design, synthesis, and evaluation of fluorogenic receptors for selective sensing of soft metal ions as well as anions and evaluation of their switching behavior.[7] In continuation of this work, we have now designed and synthesized chemodosimeter **2** which undergoes $Cu^{2+}$ driven hydrolysis in mixed aqueous media. The acylhydrazide moiety is introduced in receptor **2** due to its good binding affinity for transition metal ions.[6a,8] The catalytic hydrolysis of acylhydrazide moiety in chemosensor **2** is selectively induced by $Cu^{2+}$ ions to produce irreversible fluorescence turn-on changes at 534 and 575 nm ascribed to the highly fluorescent hydrazide derivative (**HZD**) exhibiting a twisted intramolecular charge transfer phenomenon and a ring opened form of the rhodamine, respectively. To the best of our knowledge, this is the first report where catalytic hydrolysis of a receptor in the presence of $Cu^{2+}$ ions produces fluorescence turn-on changes at two different wavelengths. Interestingly, the change in fluorescence emission is observed immediately after the addition of $Cu^{2+}$ ions which in turn indicates the highly reactive nature of $Cu^{2+}$ promoted hydrolysis of **2**. Furthermore, biological application of the chemodosimeter **2** is evaluated for *in vitro* detection of $Cu^{2+}$ ions in prostate cancer (PC3) cell lines.

The reduction of compound **1**[7d] with $NaBH_4$ gave desired compound **2** in 65% yield (Scheme 1, see Supporting Information (SI) p S5). The structure of compound **2** was confirmed by its spectroscopic and analytical data (see SI p S15−17). The absorption spectrum of receptor **2** (5 $\mu$M) in $CH_3CN/H_2O$ (8:2, v/v) shows two absorption bands at 238 and 275 nm with a shoulder at 300 nm (see SI p S6). On addition of $Cu^{2+}$ ions (0−36 equiv) the bands at 238 and 275 nm show significant enhancement with the simultaneous appearance of two new absorption bands at 394 and 556 nm. The addition of other transition and alkali metal ions did not alter the absorption spectrum of receptor **2** (see SI p S6). From the absorbance spectra of receptor **2** in the presence of $Cu^{2+}$ ions, we expected that the system might exhibit the phenomenon of resonance energy transfer during the fluorescence studies. However, the **2**-$Cu^{2+}$ system in aqueous media did not undergo any energy transfer. The fluorescence spectrum of receptor **2** (1 $\mu$M) did not exhibit any emission when excited at 380 nm in $CH_3CN/H_2O$ (8:2, v/v; Figure 1A). Upon addition of increasing amounts of $Cu^{2+}$ ions (0−22 $\mu$M) a remarkable enhancement in emission intensity was observed at 534 nm with the appearance of green colored fluorescence (Figure 1A). On the other hand, receptor **2** undergoes fluorescence enhancement at 575 nm in the presence of

**Scheme 1.** Synthesis of Compound **2**




$Cu^{2+}$ ions (0−40 $\mu$M) in $CH_3CN/H_2O$ (8:2, v/v) when excited at 540 nm due to the ring opening of the spirolactam form of the rhodamine moiety (Figure 1B). These results indicate that either $Cu^{2+}$ ions are interacting with the dimethylaminovinylbenzene/rhodamine moieties independent of each other or some other process is operating in this system. To gain insight into the whole process, we studied the fluorescence behavior of **2** with $Cu^{2+}$ ions in acetonitrile. Free **2** in acetonitrile exhibits very weak charge transfer emission at 508 nm with a shoulder at 430 nm attributed to the locally excited emission when excited at $\lambda_{ex}$ = 380 nm (inset of Figure 2A). The addition

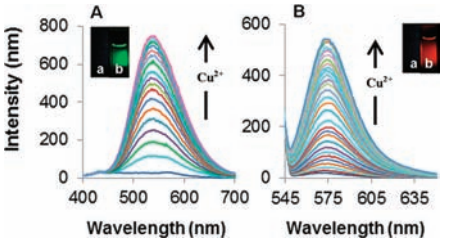



**Figure 1.** Fluorescence spectra of **2** (1 $\mu$M) in the presence of $Cu^{2+}$ ions in $CH_3CN/H_2O$ (8:2, v/v) buffered with HEPES, pH = 7.0: (A) in the presence of $Cu^{2+}$ ions (0−22 $\mu$M), $\lambda_{ex}$ = 380 nm; (B) in the presence of $Cu^{2+}$ ions (0−40 $\mu$M), $\lambda_{ex}$ = 540 nm. Insets show the fluorescence of (a) free **2** and (b) **2** + $Cu^{2+}$ ions.

of 0−10 $\mu$M of $Cu^{2+}$ ions results in the fluorescence enhancement at 508 nm along with appearance of a new emission band at 586 nm (Figure 2A). Further additions of $Cu^{2+}$ ions (11−24 $\mu$M) result in the diminishing of emission at 508 nm while the emission at 586 nm undergoes significant fluorescence enhancement (Figure 2B). These results indicate that there is a resonance energy transfer mechanism operating between the dimethylaminovinylbenzene and rhodamine moieties on addition of $Cu^{2+}$ ions in acetonitrile. The binding of $Cu^{2+}$ with an acylhydrazide moiety first induces an enhanced charge transfer from the nitrogen atom of the dimethylamino moiety to the acylhydrazide moiety which results in the enhancement at 508 nm corresponding to the donor emission (dimethylaminovinylbenzene). As the concentration of $Cu^{2+}$ ions increases, the

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phenomenon of resonance energy transfer occurs which involves energy transfer from the dimethylaminovinylbenzene moiety to the rhodamine moiety. No significant change in the fluorescence behavior was observed in the presence of other metal ions (see SI p S7). In fitting the changes in the fluorescence spectra of **2** with $Cu^{2+}$ ions in acetonitrile, the nonlinear regression analysis program SPECFIT[9] gave a good fit and demonstrated that a 2:1 stoichiometry (host:guest) was the most stable species in the solution with a binding constant (log $\beta$) = 10.30 with $\pm$ 0.07 error. The method of continuous variation[10] (Job's plot) was also used to prove the 2:1 stoichiometry (see SI p S13). The absorbance spectrum of **2** in acetonitrile (see SI p S7) is same as that observed in the aqueous media. However, the addition of $Cu^{2+}$ ions results in the appearance of new bands at 256, 354, 394, and 560 nm with a simultaneous decrease of absorbance at 238, 275, and 300 nm (see SI p S7).

The UV−vis and fluorescence behavior of **2**-$Cu^{2+}$ in acetonitrile is different from that in the mixed aqueous media. The use of water as a cosolvent along with acetonitrile does not influence the emission pattern of receptor **2**. However, the addition of $Cu^{2+}$ ions promoted the hydrolysis of receptor **2** rather than the resonance energy transfer phenomenon which results in the generation of a highly fluorescent hydrazide derivative (**HZD**) and a ring opened form of rhodamine (Figure 3). Further, to confirm $Cu^{2+}$ mediated hydrolysis, we carried out reversibility experiments in both acetonitrile and acetonitrile/water systems. The addition of diethylenetriamine (DETA) to the **2**-$Cu^{2+}$ complex in acetonitrile restored the fluorescence signal of **2** to its original level (see SI p S8). Further addition of $Cu^{2+}$ ions to the same solution gives the outcome of the **2**-$Cu^{2+}$ complex indicating the reversible behavior of **2** in acetonitrile. On the other hand, addition of DETA to the aqueous solution containing **2** and $Cu^{2+}$ ions did not alter the emission pattern of the **2**-$Cu^{2+}$ system (see SI p S9). The fluorescence emissions at 534 and 575 nm remained the same as was observed in the absence of DETA.

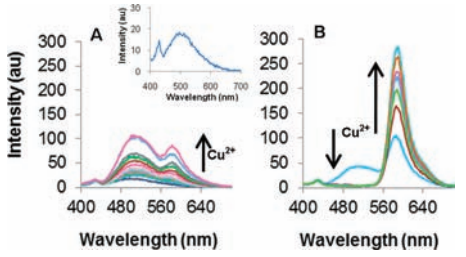


**Figure 2.** Fluorescence spectra of **2** (1 $\mu$M) in the presence of $Cu^{2+}$ ions: (A) in the presence of 0−10 $\mu$M and (B) in the presence of 11−24 $\mu$M of $Cu^{2+}$ ions. Inset shows the fluorescence spectrum of free **2**, in $CH_3CN$, $\lambda_{ex}$ = 380 nm.

The complexation of DETA with $Cu^{2+}$ ions did not affect the highly fluorescent products generated by the $Cu^{2+}$ ion promoted hydrolysis of receptor **2**, and thus the reaction of **2** and $Cu^{2+}$ ions in aqueous solution is irreversible in nature. The reaction of **2** with $Cu^{2+}$ was carried out in $CD_3CN-D_2O$ (9:1, v/v) for 5 min, and then a $^1H$ NMR spectrum of the resulting solution (see SI p S18) was recorded after eliminating the $Cu^{2+}$ ions by Chelex resin. The $^1H$ NMR spectrum shows the appearance of a singlet at 10.16 ppm corresponding to the hydroxyl group of ring opened rhodamine and a broad multiplet at 1.75−1.80 ppm corresponding to the amino protons of hydrazide derivative (**HZD**). This clearly indicates that receptor **2** was catalytically hydrolyzed in the presence of $Cu^{2+}$ ions generating highly fluorescent products, a hydrazide derivative (**HZD**) and ring-opened form of rhodamine (Figure 3). The twisted intramolecular charge transfer (TICT) state is responsible for the emission observed at 534 nm which is confirmed by observing the fluorescence emission of receptor **2** with $Cu^{2+}$ ions by varying the polarity

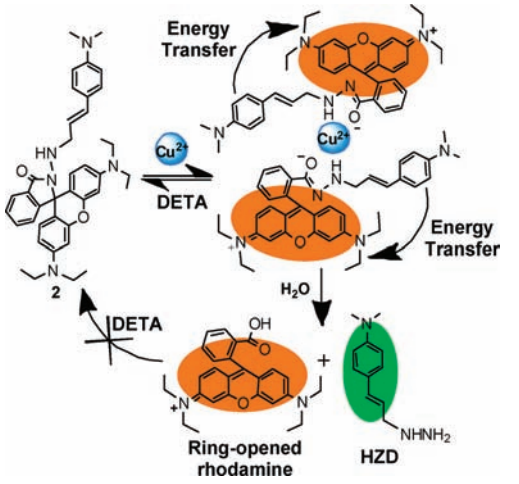


**Figure 3.** $Cu^{2+}$ induced catalytic hydrolysis of **2**.

of the solvent system (see SI p S10). As we increase the water content of the solvent system $CH_3CN/H_2O$ (9:1, 8:2, and 1:1; v/v), there is a continuous red shift of the emission band confirming the twisted intramolecular charge transfer nature of its emissive state.[11] The kinetics of the fluorescence enhancement at 534 nm by $Cu^{2+}$ mediated hydrolysis of **2** was also studied by time-dependent fluorescence spectroscopy (Figure 4). The maximum fluorescence emission enhancement at 534 nm was observed within 5 min after the additions of different concentrations of $Cu^{2+}$ ions which clearly indicate the highly reactive nature of the $Cu^{2+}$ mediated hydrolysis of **2**.

To check the practical ability of receptor **2** as a $Cu^{2+}$ selective fluorescent chemodosimeter, we carried out competitive

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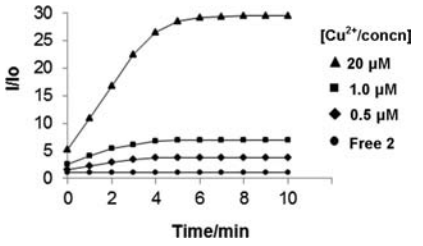


**Figure 4.** Variation of fluorescence of receptor **2** (1 $\mu$M) at 534 nm in $CH_3CN/H_2O$ (8:2, v/v) buffered with HEPES, pH = 7.0, $\lambda_{ex}$ = 380 nm in the presence of different concentrations of $Cu^{2+}$ ions ($I/I_o$; $I_o$ = initial fluorescence intensity at 534 nm; $I$ = fluorescence intensity after the addition of $Cu^{2+}$ ions at 534 nm); the fluorescence were recorded after every 1 min interval.

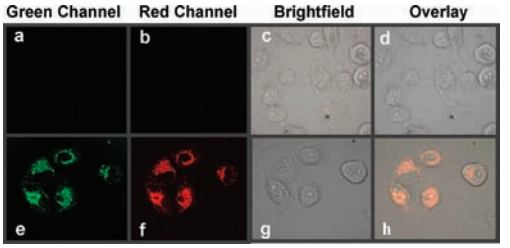


**Figure 5.** Fluorescence and brightfield images of PC3 cell lines. (a and b) Fluorescence images of cells in the green and red channels respectively treated with probe **2** (5 $\mu$M) for 20 min at 37 °C. (c) Brightfield image of (a) and (b). (d) Overlay image of (a) and (b). (e and f) Fluorescence images of cells in the green and red channels respectively upon treatment with probe **2** (5 $\mu$M) and then $Cu(ClO_4)_2$ (30 $\mu$M) for 20 min at 37 °C. (g) Brightfield image of (e) and (f). (h) Overlay image of (e) and (f), $\lambda_{ex}$ = 488 nm; fluorescence images are recorded at both green (520 ± 20 nm) and red channels (570 ± 20 nm).

experiments at two different emissions, in the presence of $Cu^{2+}$ mixed with different metal ions (see SI p S12). No significant variation in the emission was observed by comparison with or without the other metal ions. Further, the fluorescence enhancement factor for receptor **2** at 534 and 575 nm in the presence of $Cu^{2+}$ ions is increased 32- and 27-fold, respectively. The fluorescence quantum yield[12] (see SI p S3) of the **2**-$Cu^{2+}$ complex is 0.20 (at $\lambda_{em}$ = 534 nm) and 0.16 (at $\lambda_{em}$ = 575 nm) as compared to that of free **2** (0.03 and 0.01, respectively). The detection limit of **2** for $Cu^{2+}$ ions at 534 nm was found to be $20 \times 10^{-9}$ mol $L^{-1}$ (see SI p S14) which is sufficiently low and within the acceptable limit of the US EPA for the maximum allowable limit of $Cu^{2+}$ ions (1.3 ppm) in drinking water.

The potential biological application of the chemodosimeter **2** was evaluated for *in vitro* detection of $Cu^{2+}$ ions in prostate cancer (PC3) cell lines (see SI p S4). The prostate cancer (PC3) cell lines were incubated with receptor **2** (5 $\mu$M) in an RPMI-1640 medium for 20 min at 37 °C and washed with phosphate buffered saline (PBS) buffer (pH 7.4) to remove excess receptor **2**. Microscopic images showed no intracellular fluorescence which indicated that receptor **2** is nonemissive in nature (Figure 5a and b). However, after treatment with $Cu^{2+}$ ions (30 $\mu$M), the cells pretreated with receptor **2** (5 $\mu$M) show fluorescence in both the green and red channels (Figure 5e and f). The appearance of green and red fluorescence is attributed to the hydrazide derivative and ring-opened form of rhodamine produced by $Cu^{2+}$ ion mediated hydrolysis of **2**. These results suggest that receptor **2** is cell permeable and an effective intracellular $Cu^{2+}$ ion imaging agent with turn-on green and red colored fluorescence emissions.

In conclusion, we synthesized fluorescent chemodosimeter **2** which undergoes $Cu^{2+}$ ion promoted hydrolysis to produce fluorescence turn-on changes at two different wavelengths. Further, receptor **2** can also be used as a fluorescent probe for intracellular imaging of $Cu^{2+}$ ions with tunable emission (green and red) which will help in the understanding of biological processes at the molecular level.

**Acknowledgment.** We are thankful to CSIR (New Delhi) for financial support (Ref. No. CSIR Scheme 01 (2167)07/EMR-II) N. K. is thankful to CSIR (New Delhi) for junior research fellowship and Guru Nanak Dev University for providing the research facility.

**Supporting Information Available.** Experimental data and synthetic detail of compound **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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