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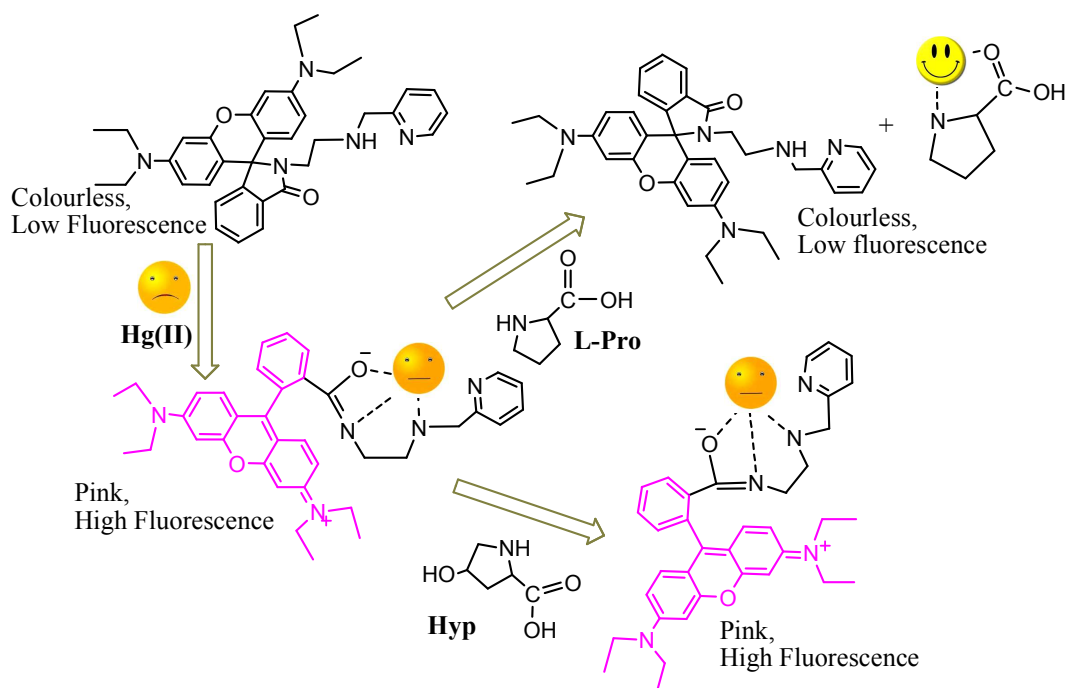
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Rhodamine based “off-on” probe for selective detection of Hg(II) and subsequent L-Proline and 4-Hydroxyproline discrimination.

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The Rhodamine derivatized probe **2** selectively coordinates with Hg(II) ion to exhibit dual mode “turn-on” signaling, subsequently ‘turned-off’ with addition of L-Proline, specific among various amino acids due to its favorable coordination environment.

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Rhodamine based “off-on” probe for selective detection of Hg(II) and subsequent L-Proline and 4-Hydroxyproline discrimination.

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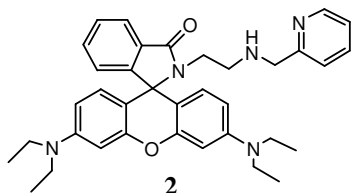
A substituted rhodamine based bifunctional chemosensor 2 showed “turn-on” chromogenic and fluorogenic dual mode signal modulation upon selective binding with Hg(II) ion whereas its Hg(II) complex selectively detects L-Proline among all biologically significant amino acids by quenching of its enhanced fluorescence. In the process of ‘Off-On-Off’ signaling, the complex therefore discriminates L-Proline and its hydroxy- derivative, which is a vital phenomena of inducing selectivity among amino acid identification.

Metals, either in neutral or ionic form, are considered as a major constituent of global pollutants. Extracted and mining from earth crust, they are being used in various industries *e.g.* as fungicides, seed preservatives etc. in agriculture, in pharmaceuticals, as pulp and paper preservatives, as catalysts in organic syntheses, in thermometers and batteries, in amalgams and in chlorine and caustic soda production. Few metals, present in certain quantities, are also essential in physiological perspective to perform various biological processes, such as functioning of critical enzymatic reactions and protein synthesis. There are few heavy metals, called *xeonobiotics* (*e.g.* lead, mercury), which hardly have any role in human physiology but are toxic¹ even at trace level of exposure. The U.S. Agency for Toxic Substances and Disease Registry (ATSDR) has ranked Hg(II) ion in second position according to its prevalence and severity of their toxicity. As an environment pollutant, mercury dispersion through atmospheric deposition has increased markedly through waste ignition; interestingly, the medical industry is one of the largest contributor² to mercury pollution. Exposure to high levels of metallic, inorganic, or organic form of mercury lead to severe health hazards³ in human being such as permanent damage to brain, kidneys, liver and developing fetus.^{3d} Therefore development of highly selective and sensitive sensors for detection of Hg(II) ion in trace level is essential for environmental monitoring and human health perspective. Other modes of their detection⁴ have various drawbacks due to high expenses, sophisticated instrumentation, complicated sample preparation procedures, along with inherent sensitivity issues. Molecular fluorescence sensors, due to its advantages in sensitivity, quick response time, non-evasive non-destructive nature and easy sample preparation, have proven to be an important tool for quantitative analysis of Hg(II) ion,

particularly in molecular biology⁵ for studying details of metal toxicity and in environmental analysis. Many fluorescence sensor based on small molecules,⁶ short peptides,⁷ gold nano particle,⁸ QDs⁹ have been reported for detection trace amount of Hg(II) ion. On the other hand, chromogenic molecular probes offer a visual perception of photophysical changes for their detection as a function of colour change. Combining together, chemosensors with ability to undergo dual mode signaling are certainly advantageous in detection of metal ions. Chemosensors based on rhodamine dyes¹⁰ have already proven to be suitable in executing dual mode signaling action to detect various metal ions in trace quantities through their excellent yet contrasting structure-function correlation.

Further, detection of trace amount of amino acids is of enormous importance in diagnostic perspective. Commonly practiced analytical procedures like spectroscopic,¹¹ chromatographic¹² or electrochemical approaches¹³ for amino acid detection have several limitations. Recent advances¹⁴ in amino acid sensing have focused on usage of fluorescent and colorimetric methods for their selective and discriminative detection, owing to methodological and mechanistic advantages. In this context, only few xanthene dye based chemosensors have been reported¹⁵ for detection of various amino acids. Such an amino acid L-Proline (**L-pro**), a primary constituent of collagen and many other proteins,¹⁶ is used as osmoprotectant in many pharmaceutical, biotechnological applications and as organo-catalyst, as precursor to many reactions etc., and hence, its identification and selective detection is highly desired. Few QD based electro-chemiluminescence probes¹⁷ has been reported for **L-pro** detection. Although a fluorescence chemosensor^{15c} for detection of N-Boc-protected proline is known, no chemosensors incorporating rhodamine has been reported so far for selective detection of **L-pro**. As discussed, rhodamine based probes provide synthetic, methodological and operational advantages in chemosensing applications, as well explore the possibility of detection of anions and neutral molecules using probe metal ion complex in a displacement approach.^{15b,18} We report herein the photo-physical signal modulation in a rhodamine B based bifunctional chemosensor **2**, which not only exhibited selectivity in detection of Hg(II) ions in aqueous medium, but also its Hg(II) complex efficiently responded towards selective detection of **L-Pro** in presence of various

amino acids. Importantly, **2**-Hg(II) complex is capable of discriminating **L-pro** in presence of its hydroxyl derivative, 4-hydroxy Proline (**Hyp**) which find a greater prospective as analytical method in various biological and industrial applications.



Scheme 1 : Graphical representation of probe **2**.

Condensation of aminoethyl-rhodamine **1**¹⁹ with 2-pyridine carboxaldehyde in EtOH followed by reduction with NaBH₄ afforded the reduced Schiff base compound **2** (See ESI), which was characterized (ESI) through ¹H-NMR, ¹³C-NMR and ESI-MS spectroscopic techniques. The colorless solution of probe **2** in MeCN-H₂O (1:1 v/v, Tris-HCl, pH 7.2) solution (conc. = 1 × 10⁻⁴ M) did not show any observable absorption in 500-700 nm region inferring to spirolactam conformation of rhodamine, which was also confirmed by appearance of the characteristic peak at ~65 ppm in ¹³C-NMR spectra in CDCl₃ (66.27 in CD₃CN-D₂O, 1:1 v/v).

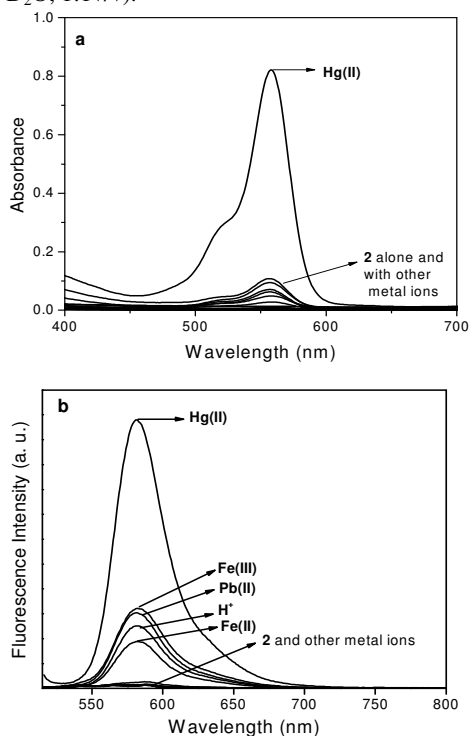


Figure 1 Absorption (a) and fluorescence (b) spectra of **2** upon addition of various metal ions (10 eq.) or H⁺ in MeCN-H₂O (1:1, v/v). Conc. [**2**] = 1 × 10⁻⁴ M (abs), 1 × 10⁻⁶ M (em), λ_{ex} = 500 nm, RT.

To investigate photo-physical spectroscopic changes of probe **2** in presence of various metal ions, aqueous solutions of metal perchlorate salts were added to its solution (MeCN:H₂O, 1:1 v/v, Tris-HCl buffer, pH 7.2), then subsequent absorption and steady state fluorescence spectra were recorded. Probe **2** did not show any enhancement in either absorption or emission intensity on addition of Li(I), Na(I), K(I), Ca(II), Ba(II), Mn(II), Mg(II), Co(II), Ni(II), Zn(II), Cd(II) ions. However, when Hg(II) was added to the colorless solution of **2**, it turned pink in colour along with a high absorption around 557 nm and fluorescence enhancement due to Hg(II) ion induced spirolactam ring opening (Fig. 1). The coordination of Hg(II) with **2** promotes its spirolactam ring opening in MeCN-aqueous medium to result in observable and appreciable spectroscopic changes; which is in good agreement with other substituted amino-ethyl-rhodamine based^{19b,20} probes. The competitive parameters between probe-metal interactions against hydration of metal ion determine the coordination module, in turn, spectroscopic signaling pattern here. In case of metal ions except Hg(II), an aqueous environment in the solvent preferably lead to their hydration over their complexation with **2**, while Hg(II) favorably coordinates to **2** in comparison to its hydration and facilitates ring opening of rhodamine to induce photo-physical changes.

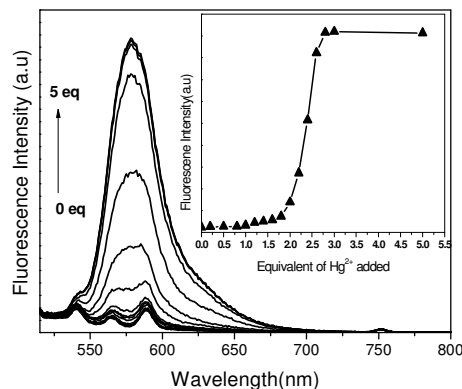


Figure 2 Fluorescence titration spectra of **2** in MeCN-H₂O (1:1 v/v) (Conc. **2** = 1 × 10⁻⁶ M, λ_{ex} = 500 nm, RT) with Hg(II) ion.

The observed increase in absorption (40-50 fold) transition of **2** around 557 nm on addition of few other metal ions such as Cr(III), Fe(III), Fe(II), Cu(II) and Pb(II) is much less in comparison to that of Hg(II) ion (~500 fold), which promote the rhodamine spirolactam ring to open and turning the colour of the solution from colourless to pink rendering a chromogenic signal selectively and sensitively in it with Hg(II) ion. Fluorescence emission intensity of **2** significantly increased (~120 fold) up on addition of Hg(II) as compare to that on addition of Cr(III), Fe(III), Fe(II), Cu(II) and Pb(II) ions (20-30 fold, Fig. 1b). Using continuous variation method (Job's Plot), the stoichiometry of the complexes were determined to be in 1:1(**2**:M(II)) ratio. The fluorescence titration pattern of **2** with Hg(II) ion lead to a sigmoidal curve (Fig. 2), exhibiting an increasing trend of fluorescence intensity with increase in concentration of Hg(II) ion up to 3 eq. and remained constant

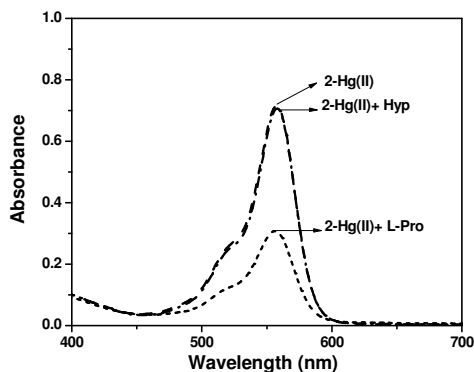


Figure 6 Absorption spectra of **2-Hg(II)** upon addition of **L-Pro** and **Hyp** in MeCN-H₂O (1:1 v/v), [2] = 1 × 10⁻⁴ M.

When **Hyp**, mostly occurring substituted **L-Pro**, was added to pink coloured solution of *in-situ* formed **2-Hg(II)**, the colour and its absorption/ fluorescence intensity were observed to remain unaffected. Although crystallographic structural evidences on complexes of **L-Pro/Hyp** with Hg(II) is not apparent, correlation to thermodynamic parameters of their complexes²¹ with other 3d-metal ions infers that **2-Hg(II)** complex rendered favorable spatial disposition and orientation for effective **L-pro** coordination with Hg(II). The steric hindrance due to presence of an hydroxyl group and conformational²² variation possibly also restricted **Hyp** coordination with Hg(II) ion and thus, fails to induce optical spectroscopic changes to that of **2-Hg(II)** complex. The different spectral behavior of the complex observed towards **L-Pro** and **Hyp** in solution thus able to discriminate between these two structurally-symmetrical biologically important amino acids.

In summary, the rhodamine B derivatized probe **2** exhibited high selectivity in coordination preference with Hg(II) ions over coexisting metal ions to result in 'turn-on' signaling pattern. The absorption and fluorescence intensity of its Hg(II) complex quenched along with a colour transition from pink to colourless upon addition of only **L-pro** among different amino acids investigated. Further, the contrast photophysical signaling behavior of **2-Hg(II)** complex in presence of **L-pro** and **Hyp** allows it to discriminate between these two derivatives. Such induction of selectivity in signaling with metal-probe complex should encourage methodological design of chemosensory probes for selective and quantitative detection of various amino acids and other small molecules of biological and environmental interest. We are presently working along this direction.

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Notes and references

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- † Electronic Supplementary Information (ESI) available: Absorption and fluorescence spectral measurements, synthetic procedure of probes and spectroscopic characterization. See DOI: 10.1039/b000000x/
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