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Graphical Abstract

Ratiometric fluorescence chemosensor based on Tyrosine derivatives for monitoring mercury ions in aqueous solutions

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Abstract

Ratiometric fluorescent chemosensors, **1** and **2** were synthesized based on Tyrosine amino acid derivatives with a pyrene fluorophore. **1** and **2** displayed high selectivity for Hg(II) ions among 13 metal ions in aqueous solutions. Both **1** and **2** sensitively detected Hg(II) ions in aqueous solutions by ratiometric response without interference of any other tested metal ions including Cu(II), Cd(II), Pb(II), and Ag(I) ions. **1** and **2** had tight binding affinities (5.72×10^{13} M^{-2}, 1.15×10^{13} M^{-2}) for Hg(II) with nano–molar detection limit. The binding mode characterized with the help of organic spectroscopic data revealed that the methoxyphenyl moiety of **1** or **2** played a vital role in the coordination of Hg(II). The deprotonation of the sulfonamide group is not a critical process for the binding of mercury ions. The methoxyphenyl moiety, sulfonamide group, and the *C*–terminal amide moiety of **1** and **2** as ligands for Hg(II) played a crucial role in the stabilization of the 2:1complexes.

Key words: Fluorescent, ratiometric, sensor, selective, Hg(II), chemosensor.

Introduction

The design and synthesis of fluorescent chemosensors for the detection and quantification of low level of Hg(II) ions in aqueous solutions have received considerable attention because Hg(II) ions are most toxic and hazardous among the various heavy and transitional metal ions (HTM).[1,2] Even low concentration of Hg(II) ions in aqueous solutions could accumulated in crop, fish, and human body and could cause a wide variety of diseases such as prenatal brain damage, serious cognitive, and motion disorders.[3,4]

Several analytical techniques have been utilized for the detection of mercury ions including atomic absorption/emission spectrometry, stripping voltammetry, and inductively coupled plasma mass spectrometry.[1,5,6] These analytical techniques have shown some limitations such as tedious time consuming procedures and expensive instruments. Thus, an inexpensive and simple techniques for monitoring Hg(II) ions have been highly demanded. In recent years, fluorescence technique has received great attention because of its inexpensive instrument, high sensitivity, rapidity, and accurate detection.[1,2,7] Thus, various types of fluorescence chemosensors for Hg(II) have been reported.[8] The fluorescent chemosensors consist of a ligand–binding site (receptor), responsible for recognizing analytes and a signal transduction site (fluorophore), converting the recognition events into fluorescent signals.[8,9] The receptor part for specific target metal ions was conjugated with the fluorophores for the synthesis of chemosensors for the metal ions. A variety of scaffolds such as thiacalixarene,[8a] azines,[8b] azadiene,[8c] dioxaoctane–diamide,[8d] cyclams,[8e,f] azacrown or azathiacrown,[8g,h] thiacrown[8i] and aza–thia moieties[8j-p] have been utilized as the receptor for the detection of Hg(II). Most of these receptors consisted of soft ligands including nitrogen and sulfur for the coordination of Hg(II). On the other hand, most of these chemosensors have some limitations due to poor solubility in aqueous solutions, cross selectivity, low sensitivity, or interference with other heavy metal ions such as Cu(II), Cd(II), and Ag(I).[8a-h,o,q] Therefore, the development of new chemosensors for selectively and sensitively monitoring Hg(II) in aqueous solutions are highly challenging.

In recent years, selective and sensitive detection of Hg(II) ions in aqueous solutions has been demonstrated with chemosensors based on amino acids containing soft ligands such as tryptophan, and methionine.[10,11] Recently, we synthesized a new chemosensor based on Tyrosine that showed a selective ratiometric response to Hg(II) ions in aqueous solution as well as live cells,[12] because the X–ray crystallographic study for mercuric reductase revealed that the tyrosine residue of the binding site acted as an important ligand for Hg(II) ions.[13] Interestingly, even though the tyrosine moiety of the chemosensor did not contain soft ligands for the coordination of Hg(II), the chemosensor showed excellent selectivity and sensitivity for Hg(II) in aqueous solutions. On the other hand, the binding mode of this chemosensor was not fully understood: 1) The hydroxyl group of tyrosine moiety of the chemosensor would directly interact with Hg(II) or not because the oxide form of the tyrosine of mercuric reductase directly interacted with Hg(II) ions in the X–ray crystallographic study, as shown in scheme 1.[13] 2) The deprotonation process of the sulfonamide group of the chemosensor might

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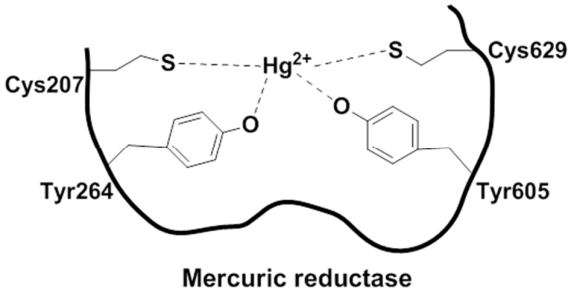
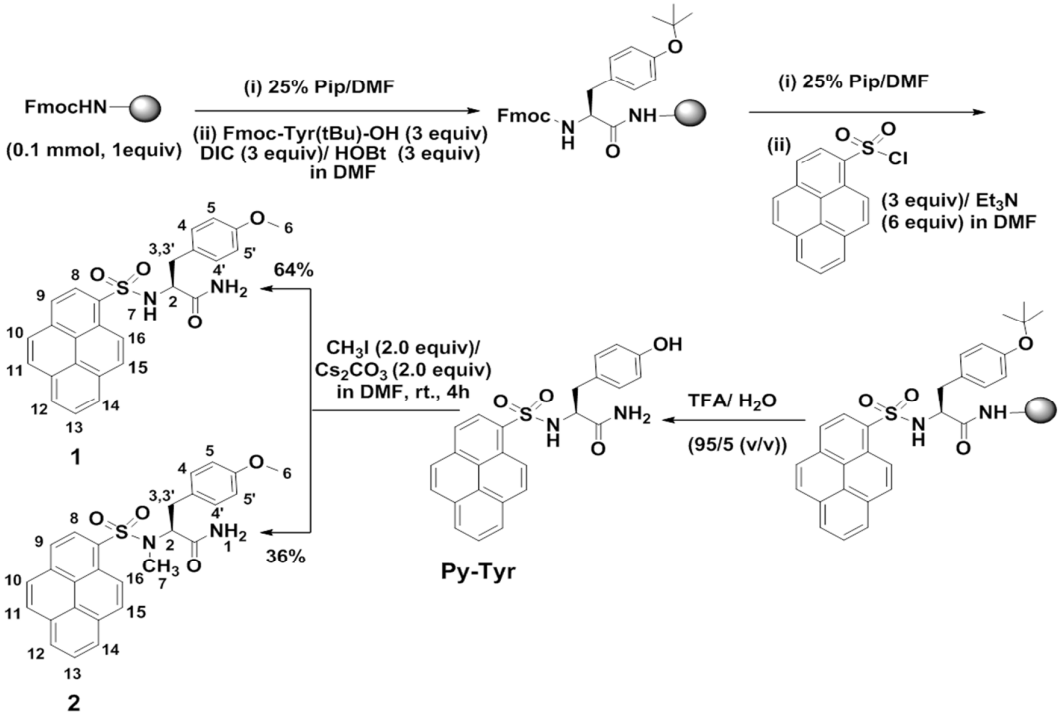
be critical for the binding of Hg(II) or not because some studies about chemosensors suggested the deprotonation process of the sulfonamide group might be important for the binding of Hg(II).[10,11]



Scheme 1 Schematic representation of the active binding site of mercuric reductase.[12]

To answer these questions about the binding mode and to design of new selective chemosensors for Hg(II), we synthesized new chemosensors **1** and **2** based on Tyrosine derivatives (Scheme 2). Interestingly, both chemosensors selectively detected Hg(II) ions in aqueous solution by ratiometric response and the binding mode of these chemosensors provided a unique function of aromatic part and sulfonamide moieties as a ligand biding site for the coordination of mercury ions and stabilized a 2:1 complex between the chemosensors and Hg(II).



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Scheme 2 Synthetic route of **1** and **2**

Results and discussion

Solid phase synthesis and characterization of 1 and 2

As shown in Scheme 2, pyrene labelled tyrosine derivatives was easily synthesized in solid phase synthesis using Fmoc chemistry.[14] Among the various fluorophores, pyrene was selected as a fluorophore because of good photophysical property such as high fluorescence quantum yield, chemical stability, and long fluorescence lifetime.[14a,b,c] Additionally, pyrene shows monomer and excimer emissions depending on the proximity between the pyrene flurophores.[14d] The intensity ratio of the excimer emission to the monomer emission is sensitive to the distance between two pyrene fluorophores[14a,d] which make it possible for ratiometric detection of target molecules if the chemosensor will form a 2:1 complex for the target molecules The yield of pyrene labeled Tyr derivatives, ($PySO_2$–Tyr(OCH_3)–NH_2, **1**) and ($PySO_2$(OCH_3)–Tyr(OCH_3)–NH_2, **2**) was 64% and 36%, respectively. The experimental procedure for the synthesis and characterization of **1** and **2** are described in the experimental procedure section (Fig. S1–S14).

Fluorescence emission optimization studies with Hg(II)

The stock solutions of **1** (1.24×10^{-3} M) and **2** (1.05×10^{-3} M) were prepared in DMSO/H_2O (1:1, v/v) and stored in a cold and dark place. The UV−Visible absorption spectra of both **1** and **2** elicits a typical pyrene absorption band at 353 nm in H_2O/DMSO (95:5, v/v, 10 mM HEPES) at pH 7.4 (Fig. S15).

The response of **1** and **2** to Hg(II) ions was characterized by fluorescence spectroscopy in mixed organic-aqueous solution at pH 7.4. The fluorescent emission response of **1** (30 μM) to Hg(II) ions was investigated in aqueous solutions (10 mM HEPES, pH 7.4) containing different volume of DMSO (Fig. 1). Interestingly, the fluorescence behavior of **1** was dependent on the volume percentage of DMSO. The fluorescence emission spectrum of free **1** measured in aqueous solution containing 0.1% DMSO displayed a strong excimer emission at 490 nm, originated from the π−π stacking between two pyrene moieties, and weak monomer emissions at 386 and 400 nm. Upon increasing the percentage of DMSO in aqueous solution (10 mM HEPES at pH 7.4), the decrease of excimer emission and the considerable increase of monomer emissions were observed. The higher percentages of DMSO (>50%) in aqueous solution (10 mM HEPES at pH 7.4) induced a strong monomer emission intensity and the very weak excimer emission intensity of free **1**.

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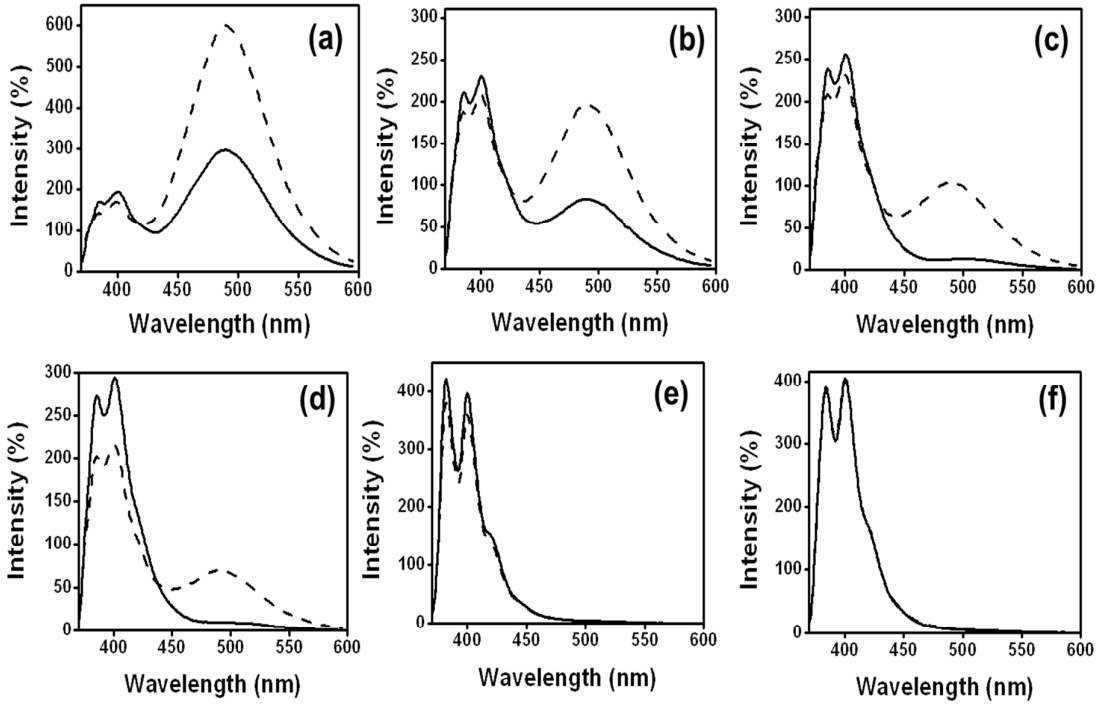

Fig. 1 Fluorescence emission intensity of **1** (30 μM) in the absence (——) and presence (----) of 2.5 equiv of Hg(II) in aqueous solution (10 mM HEPES, pH 7.4) containing DMSO (v/v) (a) 0.1, (b) 3, (c) 5, (d) 20 (e) 50 and (f) in 100% DMSO; λ_{ex}= 353 nm and slit= 15/5.0.

Upon addition of Hg(II) (2.5 equiv), the enhancement of excimer and concomitant decrease of monomer emission were observed in aqueous solutions containing 0.1%, 3%, 5%, 20% DMSO. The chemosensor **1** showed a significant ratiometric response to Hg(II) in these solvent conditions. However, a negligible enhancement of excimer and a small decrease of monomer emission were induced by Hg(II) in aqueous solution containing 50% DMSO. There was no considerable change of fluorescent spectrum in 100% DMSO solution. This indicates that the formation of a 2:1 complex of **1** and Hg(II) depended on the solvent polarity and hydrophobic interactions played an important role in the formation of a 2:1 complex. Considering the ratiometric response to Hg(II), aqueous solution containing 5% DMSO (H_2O/DMSO, 95:5, v/v, 10 mM HEPES at pH 7.4) was chosen as the solvent system for further studies.

Fluorescence emission response to metal ions

As shown in Fig. 2, the fluorescence emission spectra of **1** in the absence of metal ions in aqueous solution displayed typical emission intensities at 386 and 400 nm, attributed

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to the pyrene monomeric emission (H_2O/DMSO, 95:5, v/v, 10 mM HEPES at pH 7.4). Upon addition of Hg(II), the monomer emission intensities at 386 and 400 nm considerably decreased and concomitant increase of pyrene excimer emission at 490 nm was observed. Interestingly, chemosensor **1** did not show any response to other tested metal ions including Na(I), K(I), Mg(II), and Al(III) as chloride anion and Ca(II), Co(II), Cr(III), Fe(III), Cu(II), Cd(II), Pb(II), Ag(I) and Zn(II) as perchlorate anion.

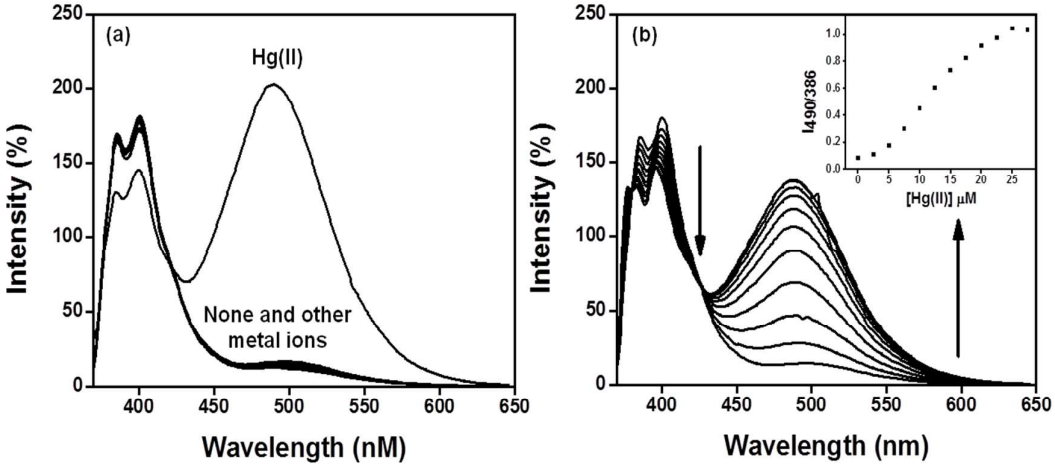

Fig. 2 Fluorescence emission spectra of **1** (40 μM) (a) in the presence of various metal ions (40 μM) except Na(I), K(I), Mg(II), and Mg(II) which were used 2 mM, (b) upon gradual addition of Hg(II) (0, 0.0625, 0.125, 0.1875, 0.250, 0.3125, 0.375, 0.4375, 0.500, 0.5625, 0.625, and 0.6875 equiv) in aqueous solution (H_2O/DMSO, 95:5, v/v, 10 mM HEPES at pH 7.4), (λ_{ex}= 353 nm, slit 15/5).

Fig. 2b exhibits the gradual emission intensities change of **1** upon addition of Hg(II). The gradual addition of Hg(II) to the solution of **1** resulted in the considerable decrease of the pyrene monomer emission intensities at 386 and 400 nm and concomitantly increase of the pyrene excimer emission at 490 nm. This was mainly due to the pyrene dimerization (π−π stacking) of the chemosensors in the presence of Hg(II). The intensity ratio (I_{490}/I_{386}) between excimer and monomer emission changed from 0.085 to 1.047 (ca. 12.3 fold enhancement) upon the addition of about 0.6 equiv of Hg(II) (Fig. 2b, inset). Similarly, **2** also showed selective ratiometric response to Hg(II) among the various tested metal ions (Fig. 3a). The intensity ratio (I_{486}/I_{385}) between excimer and monomer emission was enhanced by 6.45 fold. About 0.75 equiv of Hg(II) was required for the saturation of the intensity ratio change (Fig. 3b, inset). These results suggested that both **1** and 2 showed sensitive and selective ratiometric responses to Hg(II) in aqueous solution (H_2O/DMSO, 95:5, v/v, 10 mM HEPES at

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pH 7.4).

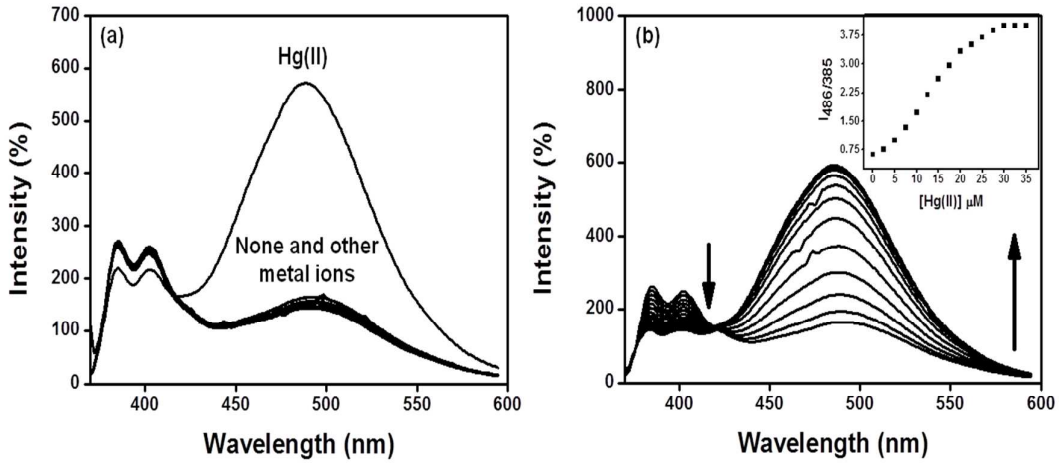

Fig. 3 Fluorescence emission spectra of **2** (40 μM) (a) in the presence of various metal ions (40 μM) except Na(I), K(I), Mg(II), and Mg(II) which were used 2mM, (b) upon gradual addition of Hg(II) (0, 0.0625, 0.125, 0.1875, 0.250, 0.3125, 0.375, 0.4375, 0.500, 0.5625, 0.625, 0.6875, 0.75, 0.8125, and 0.875 equiv) in aqueous solution (H_2O/DMSO, 95:5, v/v, 10 mM HEPES at pH 7.4; λ_{ex}= 353 nm, slit 15/6).

The pyrene dimerization of the chemosensors **1** and **2** in the presence of Hg(II) was further confirmed by UV–Visible absorption (Fig. S16). The absorbance band at 353 nm decreased with the gradual addition of Hg(II). This was due to the formation of dimerization between two pyrene moieties in the ground state in the presence of Hg(II).[9a,10b,11]

Binding stoichiometry and binding affinity

We investigated the binding stoichiometry between the chemosensors (**1** and **2**) and Hg(II) by using Job's plot analysis (Fig. S17). A maximum intensity around at 0.4 mole fraction in Job's plot analysis indicates that both **1** and **2** may form a 2:1 complex with Hg(II), respectively in aqueous solution (H_2O/DMSO, 95:5, v/v, 10 mM HEPES at pH 7.4). By assuming a 2:1 complex formation, the association constants (K_a) of **1** and **2** for Hg(II) were calculated as 5.72×10^{13} M^{-2} (R^2 = 0.95) and 1.15×10^{13} M^{-2} (R^2 = 0.97), respectively (Fig. S18).[12,16] The values clearly indicate that **1** and 2 have potent binding affinities for Hg(II) in aqueous solution. The sensitivity of **1** and **2** for Hg(II) was determined based on the linear relationships between the maximum monomer emission intensity at 386 nm and the concentration of Hg(II) (Fig. S19). The detection limit was calculated as 22.2 nM (R^2 = 0.99) and 44.0 nM (R^2 = 0.99) for **1** and **2**, respectively by using **3σ/m**, where **σ** was the standard

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deviation of the blank measurements, and **m** was the slope (sensitivity) of the intensity at 386 nm versus concentration of Hg(II) in the plot. The result indicates that **1** is more sensitive than **2**. Both chemosensors **1** and **2** can be useful to detect qualitatively low levels of Hg(II) in aqueous solution.

Interference effect of other metal ions on fluorescence emission

As shown in Fig. 4, we investigated the interference effect of other metal ions on the detection ability of chemosensor **1** and **2** for Hg(II). The emission intensity ratio of **1** and **2** in the presence of Hg(II) were not considerably affected by the presence of high concentration (2 mM) of Group I, II, and III metal ions such as Na(I), Mg(II), and Al(III). The intensity ratio of **1** and **2** in the presence of Hg(II) was not changed by other transition metal ions (1 equiv). Interestingly the heavy and transition metal (HTM) ions such as Ag(I), Pb(II), Cd(II), Zn(II), and Cu(II) (1 equiv) did not interfere with the detection of **1** and **2** for Hg(II), respectively. Most of the reported chemosensors for Hg(II) suffered from the cross sensitivity with other heavy and transition metal ions such as Cu(II), Ag(I), Cd(II), and Pb(II).[8a-h,o,q,11b], whereas both **1** and **2** were highly selective for Hg(II) among other metal ions.

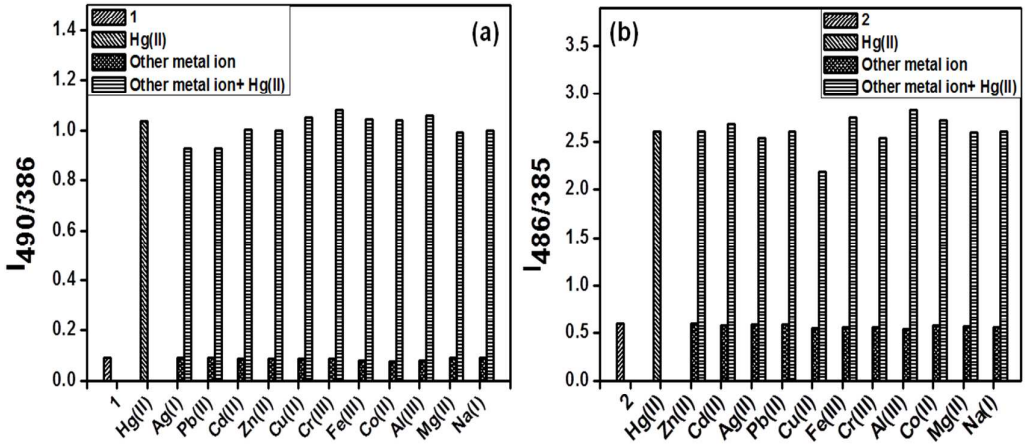

Fig. 4 Fluorescence emission intensity ratio of (a) **1** (I_{490}/I_{386}, 40 μM, slit 15/5) and (b) **2** (I_{486}/I_{385}, 40 μM, slit 15/6) in the presence various other metal ions and of Hg(II) (1 equiv) in aqueous solution (H_2O/DMSO, 95:5, v/v, 10 mM HEPES at pH 7.4). The concentration of Group I, II, and III metal ions were 2 mM, respectively and the concentration of other metal ions were 40 μM.

Fluorescence emission studies at different pH

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The ratiometric response of **1** and **2** to Hg(II) was examined at different pH to investigate the working pH range of the chemosensors and the role of the functional groups of **1** and **2** for the binding of Hg(II). The detection of HTM in acidic conditions is highly desirable because the solubility of HTM may increase in acidic conditions so the contamination of HTM to environment is more serious in acidic conditions. However, many of the reported chemosensors including ratiometric fluorescent chemosensors were not able to detect Hg(II) ions in acidic conditions. This was mainly due to the inhibition of PET or ICT process for sensing the metal ions by the protonation of amine group of the chemosensors in acidic conditions.[8b-f,9,]

As shown in Fig. 5, both **1** and **2** showed considerable ratiometric response to Hg(II) in acidic pH. The emission intensity ratio increased from 0.08 to 0.34 for **1** and 0.54 to 1.02 for **2** in the presence of Hg(II), respectively. Under basic conditions (pH = 10.5 or 11.5), **1** displayed a ratiometric response to Hg(II). The emission intensity ratio (I_{490}/I_{386}) of **1** increased from 0.1 to 3.4 in the presence of Hg(II) at pH 10.5, whereas the intensity ratio increased from 0.09 to 3.1 at pH 11.5. The deprotonated sulfonamide group ($pK_a \approx 10$) did not considerably affect the ratiometric response to Hg(II).[10d,11c] Under basic condition, chemosensor **2** also showed a ratiometric response to Hg(II). Even though **2** containing *N*–methyl sulfonamide group showed a more enhanced excimer emission in the presence of Hg(II) than **1**, the intensity ratio change of **2** (from 0.28 to 2.82) induced by Hg(II) was slightly lower than that of **1**. The pH titration experiment reveals that **1** and **2** are suitable for monitoring Hg(II) by ratiometric response over the wide range of pH values (pH4.5–11.5).

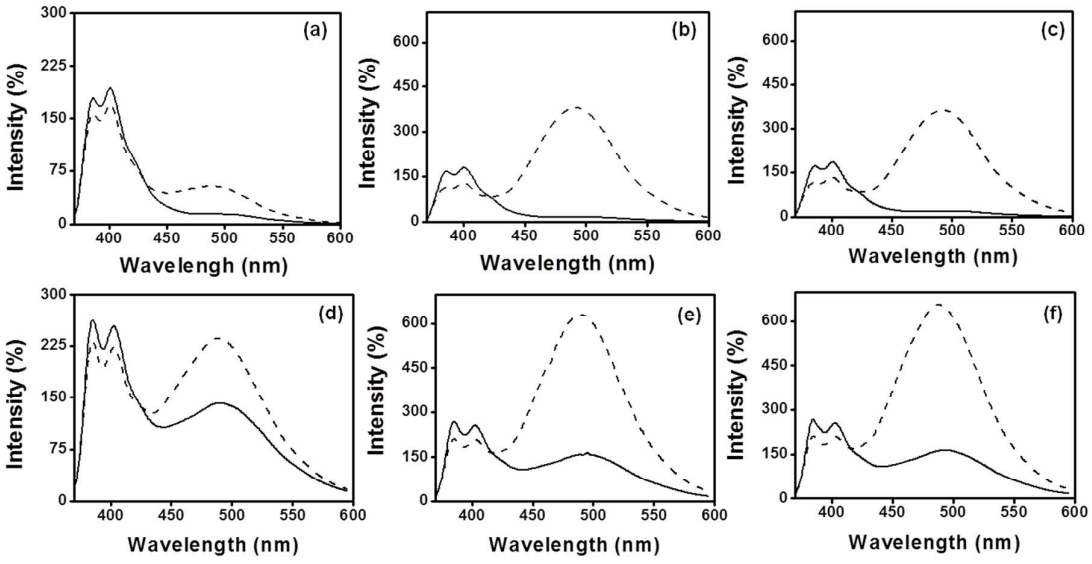

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Fig. 5 Fluorescence spectra of **1** (40 μM) in the absence (——) and presence (-----) of Hg(II) (1.0 equiv) at various pH (a) 4.5, (b) 10.5, and (c) 11.5 and fluorescence spectra of **2** (40 μM) in the absence (——) and presence (-----) of Hg(II) (1.0 equiv) at various pH (d) 4.5, (e) 10.5, and (f) 11.5.

Binding mode of 1 and 2 with Hg(II)

The binding mode of the chemosensor with Hg(II) was investigated by using ESI mass spectrometry. When 1.0 equiv of Hg(II) was added to the solution of **1** (500 μM), a new peak appeared at 1114.74 (*m*/*z*) corresponding to $[(2\cdot\mathbf{1}) + Hg^{2+} - H^{+}]^{+}$ (Fig. S20). This results clearly suggests that **1** may form a 2:1 complex between **1**–Hg(II). Similarly, chemosensor **2** also may form a 2:1 complex upon addition of 1.0 equiv of Hg(II) because the peak at 1145.09 (*m*/*z*) corresponding to $[(2\cdot\mathbf{2}) + Hg^{2+} - H^{+}]^{+}$ appeared (Fig. S21). The result suggests that both chemosensors may form a 2:1 complex, respectively.

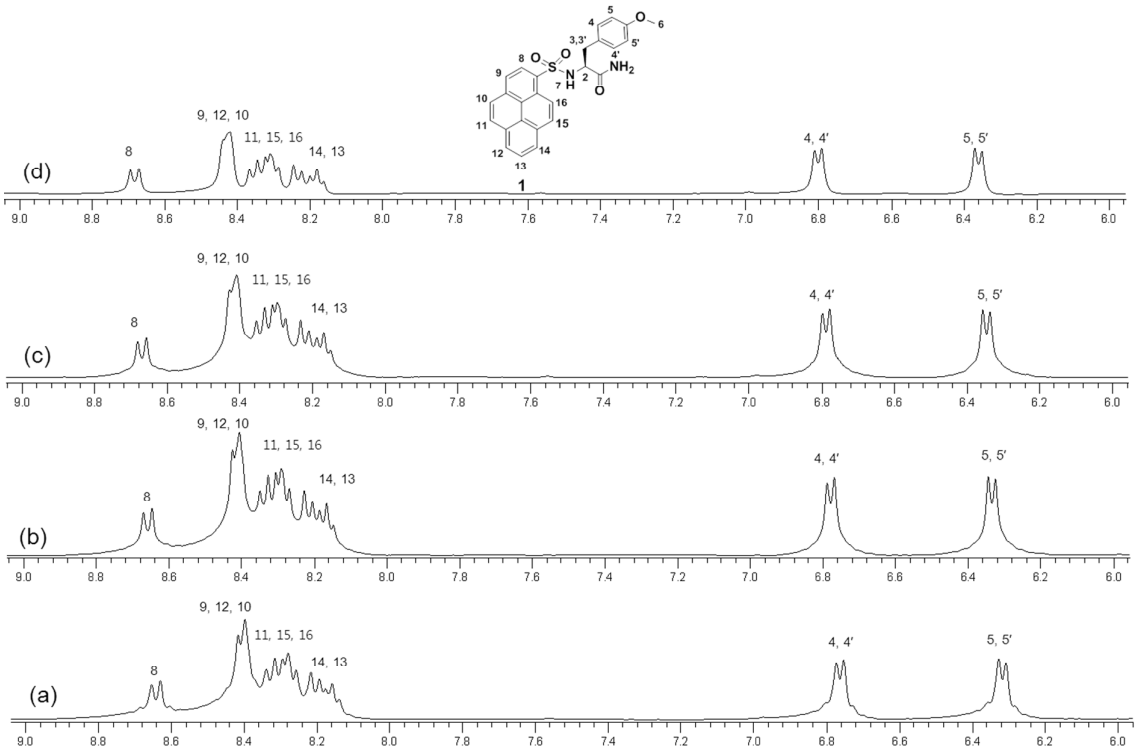

Fig. 6 Partial ^{1}H NMR spectra (400 MHz) of chemosensor **1** (8.4 mM) with (a) 0 equiv of $Hg(ClO_4)_2$, (b) 0.25 equiv of $Hg(ClO_4)_2$, (c) 0.50 equiv of $Hg(ClO_4)_2$, and (d) 1 equiv of $Hg(ClO_4)_2$ in D_2O/DMSO–d_6 (8:2, v/v, pH ≅7.5)

^{1}H NMR titration experiments provided additional information for the detailed binding mode of **1** and **2** with Hg(II), respectively. ^{1}H NMR spectra were recorded in D_2O/DMSO–d_6 (8:2, v/v) at pH 7.5 adjusted with 1% NaOD because both chemosensors

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exhibited much better ratiometric response to Hg(II) in neutral and basic pH rather than acidic pH. As depicted in Fig. 7, the ^{1}H NMR spectra of **1** recorded with increasing amount of Hg(II) displayed the downfield shift (Δ0.02 and Δ0.05 ppm) of proton signals at H–4,4′ and H–5,5′ corresponding to the methoxyphenyl moiety. These shifts were attributed to the coordination of Hg(II) with the methoxyphenyl moiety of **1**. The downfield shifted (Δ0.04 ppm) of the proton signal (H-8) at 8.64 ppm of the pyrene near (*ortho*) to the sulfonamide group was noticed, which was due to the interactions between Hg(II) and the sulfonamide group. Subsequently, all other aromatic protons of the pyrene were also slightly downfield shifted upon binding with Hg(II).

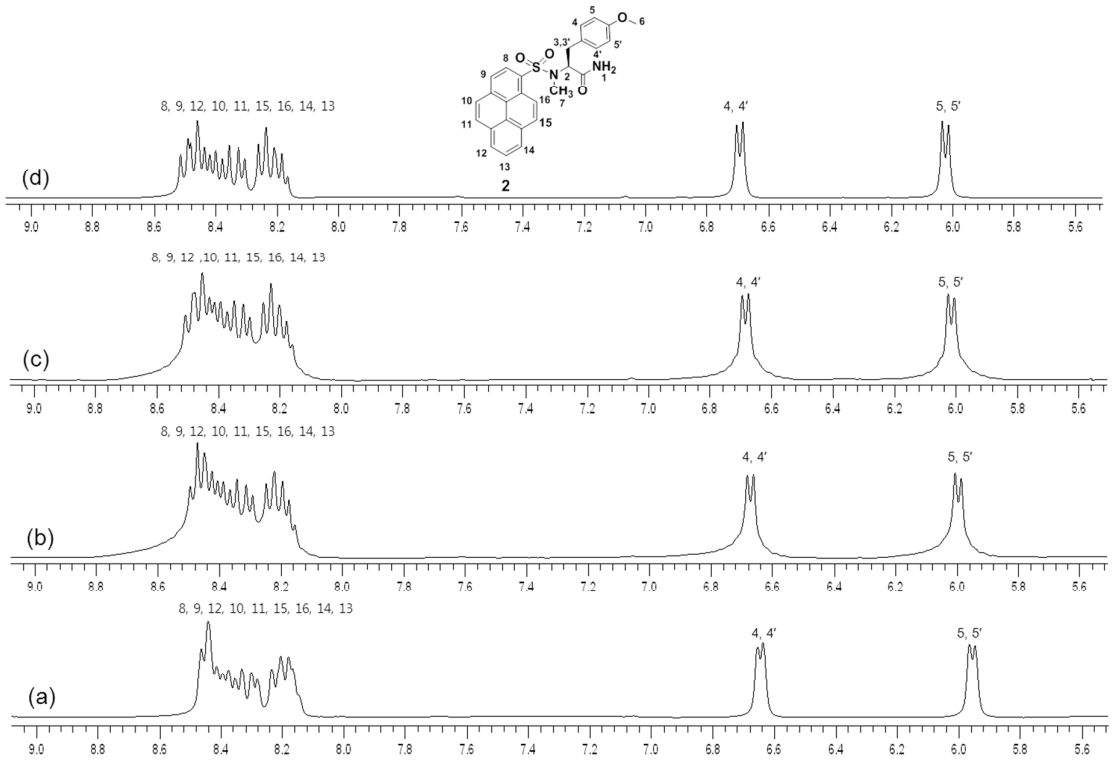

Fig. 7 Partial ^{1}H NMR spectra (400 MHz) of chemosensor **2** (8.7 mM) in (a) 0 equiv of $Hg(ClO_4)_2$, (b) presence of 0.25 equiv, (c) presence of 0.50 equiv, and (d) presence of 1 equiv of $Hg(ClO_4)_2$ in D_2O/DMSO–d_6 (8:2, v/v, ~pH = 7.5)

As shown in Fig. 8, the binding mode of **2** and Hg(II) was also investigated by ^{1}H NMR titration. After the addition of 1 equiv Hg(II) induced downfield shift (Δ0.05 and Δ0.07 ppm) of proton signals for H–4,4′ and H–5,5′ corresponding to the methoxyphenyl protons, respectively. This indicates that Hg(II) may strongly chelate the methoxyphenyl moiety of **2**. The slightly downfield shifted of all aromatic protons of the pyrene were also observed in the

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presence of Hg(II). This may be due to the interactions of the *N*–methyl sulfonamide group with Hg(II). This result strongly suggests that the deprotonation process is not an important criteria for the binding of Hg(II) ions. ^{1}H NMR indicates that methoxyphenyl and the sulfonamide moieties acted as ligands for coordination of Hg(II). The *C*–terminal amide proton peaks of the **1** and **2** could not be observed due to D_2O in this solvent system, however the previous results about the chemosensors based on amino acid suggest that *C*-terminal amide group play an important role in the binding of target metal ions.[10b,11a]

In chemosensor **1** showed a potent binding affinity for Hg(II) compared to **2** in aqueous solution. The association constants (K_a) of **1** and **2** for Hg(II) were 5.72×10^{13} M^{-2} and 1.15×10^{13} M^{-2}, respectively. These values clearly indicate that **1** and **2** have more potent binding affinities for Hg(II) than the previously reported chemosensor (**Py–Tyr**) based on tyrosine (K_a, 3.5×10^{12} M^{-2}) in aqueous solution.[12] These results clearly suggest that the oxide form of tyrosine moiety of **Py-Tyr** may not directly interact with Hg(II) like the binding mode elucidated in the X–ray crystallographic study.[13] Furthermore, the more potent binding affinity of **1** than that of **Py-Tyr** strongly suggests that the methoxyphenyl moiety of **1** as an important ligand may chelate Hg(II). Interestingly, chemosensor **2** containing *N*–methyl sulfonamide group (–SO_2–NCH_3–) showed a similar binding affinity as chemosensor **1** containing sulfonamide group, Which suggests that the deprotonation process is not a crucial for the binding of Hg(II) ions. The binding mode of both **1** or **2** with Hg(II) was proposed based on fluorescent and UV–visible spectra, pH titration result, ESI-mass spectra and ^{1}H NMR titration result, and the previously reported binding mode of chemosensors based on amino acids.[10b,11a] As shown in Scheme 3, the chemosensors formed a 2:1 complex with Hg(II) by chelation of the sulfonamide group, the amide group, and the methoxyphenyl moiety. Hg(II) may interact with the methoxyphenyl moiety maybe by cation–pi interactions and the methoxy as an electron donating group increased electron density of the aromatic part. The binding mode was consistent with the binding mode proposed by *Li et al* using X–ray crystal structure between dansyl–tryptophanmethyl ester and Hg(II),[11a] in which the sulfonamide and ester groups of the chemosensor were important ligands for stabilizing a 2:1 complex between the chemosensor and Hg(II).

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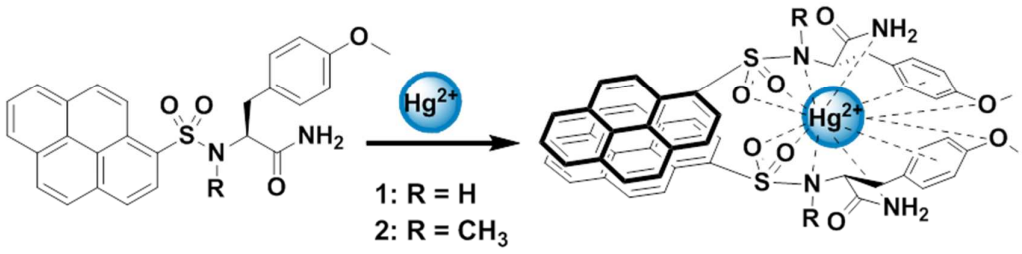

Scheme 3 A proposed binding mode of chemosensor **1** or **2** with Hg(II)

Conclusions

The chemosensors **1** and **2** based on Tyrosine derivatives detected selectively Hg(II) ions in aqueous solution by ratiometric response. **1** and **2** had potent binding affinities (5.72×10^{13} M^{-2} and 1.15×10^{13} M^{-2}) for Hg(II) in aqueous solutions and the sensitive ratiometric response to Hg(II) was not interfered by any other tested metal ions including Ag(I), Pb(II), and Cu(II). The detection limit of **1** and **2** for Hg(II) in aqueous solutions was determined as 22.2 nM and 44.0 nM, respectively. The methoxyphenyl moiety of **1** or **2** played a vital role in the coordination of Hg(II). The deprotonation of the sulfonamide group (–SO_2–NH–) was not a critical process for the binding of mercury ions. The methoxyphenyl moiety, sulfonamide group, and the *C*–terminal amide moiety of **1** and **2** as ligands for Hg(II) played a crucial role in the stabilization of the 2:1complexe.

Experimental Section

Reagents

Rink Amide MBHA resin, Fmoc-Tyr(tBu)–OH, *N,N*–diisopropylcarboiimide (DIC), and 1–hydroxybenzotriazole (HOBt) were purchased from Bead Tech. Other reagents for solid phase synthesis including trifluroacetic acid (TFA), triethylamine, *N,N*–dimethylformamide (DMF), piperidine, cesium carbonate (Cs_2CO_3), iodomethane (CH_3I), and anhydrous sodium sulfate (Na_2SO_4) were purchased from Aldrich. 1–Pyrenesulfonyl chloride was synthesized from 1-pyrenesulfonic acid (purchased from Aldrich).

Solid phase synthesis: General experimental procedure

Py-Tyr was efficiently synthesized in solid-phase synthesis with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Scheme 1).[13] Diisopropylcarbodiimide (DIC) and 1-hydroxylbenzotriazole (HOBt) in situ activation method was used for the coupling reactions. Fmoc–Tyr(tBu)–OH (0.3 mmol, 0.3 equiv) was loaded to Rink Amide MBHA

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(0.1 mmol, 0.1 equiv) according to the reported procedure. After washing, drying, and deprotecting the Fmoc group with 25% piperidine in *N,N*–dimethylformamide (DMF). The 1–pyrenesulfonyl chloride (0.3 mmol, 0.3 equiv) was then coupled with the deprotected amino group in the presence of triethylamine (0.6 mmol, 0.6 equiv). Finally, the cleavage of **Py-Tyr** from the resin was accomplished with CF_3COOH/H_2O (TFA/ Water, 95/5, v/v) at room temperature for 3h. Following vacuum filtration and removal of TFA with N_2 blow–off, crude product was precipitated from cold ether. The solid precipitate was centrifuged, washed with ether, and lyophilized under vacuum. The crude product was used further to synthesis of **1** and **2** in solution phase.

To a stirred solution of **Py–Tyr** in dry DMF (1.5 mL) 0.2 equiv cesium carbonate (Cs_2CO_3) were added under nitrogen atmosphere at 0 ^oC. Reaction mixture stirred for 10 min at room temperature then, 0.2 equiv Iodomethane (CH_3I) dissolved in dry DMF (0.5 mL) was slowly added by drop by drop. After completion of 4h, the reaction mixture was filtered and residue washed with DMF. To filtrate, 2 mL of water was added and extracted with ethyl acetate (3 × 3 mL). The collected ethyl acetate portions dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude product purified with semi preparative HPLC using water (0.1% TFA)/acetonitrile (0.1% TFA) gradient. The retention time of **1** and **2** are 52 and 60 min, respectively. The compound **1** and **2** were characterized by IR, ^1H and ^{13}C NMR and ESI-mass data. The melting point, IR, ^1H NMR, ^{13}C NMR, and ESI-mass data of **1** and **2** are given below.

Compound **1:** White solid, mp 255–256 °C; IR (KBr): 3448, 3328 (br s) 2917, 1680, 1510, 1323, 1120 cm^{-1}; ^1H NMR (400 MHz, $DMSO_6$) δ 9.05 (s, 1H), 8.79 (d, J = 8.4 Hz, 1H), 8.47–8.42 (m, 3H), 8.39–8.34 (m, 3H), 8.26 (d, J = 8.4 Hz, 1H), 8.18 (t, J = 8.4 Hz, 1H), 7.48 (br s, 1H), 7.00 (br s, 1H), 6.88 (d, J = 8.6 Hz, 2H), 6.46 (d, J = 8.6 Hz, 2H), 4.74 (t, J = 8.5 Hz, 1H), 3.01 (s, 3H), 2.89 (dd, J = 12.0, 2.0 Hz, 1H); 2.61 (dd, J = 12.0, 1.8 Hz, 1H; ^{13}C NMR (100 MHz, $DMSO_6$) δ 171.61, 155.8, 134.1, 131.3, 130.5, 130.1, 129.8, 129.6, 129.5, 127.5, 127.1, 127.0, 126.9, 126.8, 124.3, 124.0, 123.3, 123.1, 114.9, 59.4, 34.5, 30.4; ESI–Mass (m/z): $[M+H]^+$ at 459.18; HRMS-FAB (m/z): $[M+H]^+$ calculated for $C_{26}H_{23}N_2O_4S$: 459.1379, observed: 459.1373.

Compound **2:** White solid, mp 154–155 °C; IR (KBr): 3443, 2939, 1679, 1509, 1241, 1120 cm^{-1}; ^1H NMR (400 MHz, $DMSO_6$) δ 8.59 (d, J = 8.6 Hz, 1H), 8.50-8.43 (m, 1H),

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8.43–8.37 (m, 2H), 8.36-8.31 (m, 2H), 8.28–8.23 (m, 2H), 8.21–8.15 (m, 1H), 7.54 (br s, 1H), 7.07 (br s, 1H), 6.78 (d, J = 8.4 Hz, 2H), 6.17 (d, J = 8.4 Hz, 2H), 4.65-4.61 (m, 1H), 3.17 (s, 3H), 3.12 (s, 3H), 2.89 (dd, J = 12.8, 2.0 Hz, 1H), 2.68 (dd, J = 12.8, 1.8 Hz, 1H); ^{13}C NMR (100 MHz, $DMSO_6$) δ 172.0, 157.2, 134.1, 131.2, 130.5, 130.0, 129.5, 129.3, 129.2, 128.3, 127.4, 127.3, 126.9, 126.7, 124.3, 123.8, 123.1, 112.2, 112.8, 59.3, 54.2, 34.3, 30.4; ESI–Mass (m/z): $[M+H]^+$ at 473.19; HRMS-FAB (m/z): $[M+H]^+$ calculated for $C_{27}H_{25}N_2O_4S$: 473.1535, observed: 473.1530.

General fluorescence measurements

The stock solutions of **1** (1.23×10^{-3} M) and **2** (1.05×10^{-3} M) were prepared in DMSO/H_2O (1:1, v/v) and stored in a cold and dark place. This stock solution was used for all spectrofluoremetric experiments after appropriate dilution. The fluorescence experiments were carried out using the above referred solution after maintaining the pH of the solution to 7.4 using 10 mM HEPES buffer solution. Fluorescence emission spectrum of a sample in a 10 mm path length quartz cuvette was measured in 10 mM HEPES buffer solution at pH 7.4 using a Perkin Elmer luminescence spectrophotometer (model LS 55). Emission spectra (360–600 nm) of **1** and **2** in the presence of several metal ions (Na(I), K(I), Mg(II), and Al(III) as chloride anion and Ca(II), Co(II), Cr(III), Cu(II), Fe(III), Mn(II), Ni(II), Pb(II), and Zn(II) as perchlorate anion) were measured by excitation with 352 nm The slit size for excitation and emission were used for **1** (15 and 5 nm) and **2** (15 and 6 nm), respectively. The concentration of **1** and **2** were confirmed by UV absorbance at 342 nm for pyrene group. The molar extension coefficient (ε) of **1** and **2** is 16000 cm^{-1} M^{-1}at 342 nm.

Determination of association constant

The association constant for 2:1 complex was calculated based on the titration curve of the probes **1** and **2** with Hg(II). Association constants were determined by a nonlinear least squares fitting of the data with the following equation as referenced elsewhere.[9b,10b,11,15]

$$y = \frac{x}{2 \times a \times b \times (1-x)^2} + \frac{x \times b}{2}$$

Where $\boldsymbol{x}$ is $I–I_o/I_{max}–I_o$, $\boldsymbol{y}$ is the concentration of metal ions, $\boldsymbol{a}$ is the association constant, and $\boldsymbol{b}$ is the concentration of chemosensor.

Determination of detection limit

The detection limits were calculated based on the fluorescence titration. To

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determine the S/N ratio, the emission intensity of **1** and **2** without Hg(II) were measured by 10 times and the standard deviation of blank measurements was determined. Three independent duplication measurements of emission intensity were performed in the presence of Hg(II) and each average value of the intensities was plotted as a concentration of Hg(II) for determining the slope. The detection limit is then calculated with the following equation.

$$\textbf{Detection limit} = 3\sigma/\boldsymbol{m}$$

Where **σ** is the standard deviation of blank measurements, ***m*** is the slope between intensity versus sample concentration.

Supporting information

Further experimental details including (i) IR, ^{1}H NMR, ^{13}C NMR, ESI mass, FAB-Mass, FAB-HRMS and Uv–Visible absorption spectra, (ii) Uv–Visible titration spectra (iii) Job's plot (iv) association constant, (v) detection limits, and (vi) ESI mass of **1**–Hg(II) and **2**–Hg(II) complex data of **1** and **2** are available in the Supporting Information.

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