Synthesis and ion selectivity studies of potential fluorescent heavy metal ion indicators

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Abstract
Two new potential heavy metal ion indicators of the phenanthroline-based family, displaying distinct fluorescence profiles, were synthesized. Their ion selectivity is discussed in terms of their ionophore/fluorophore properties and the extent of conjugation in their framework. The 2-(2-hydroxyphenyl)benzazole containing fluorophores used in the construction of the probes, exhibit a unique fluorescence profile with a high Stokes shift that is attributed to an excited state intramolecular proton transfer (ESIPT).

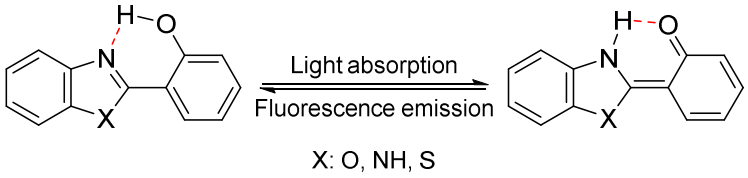
Keywords: 5-Amino-1,10-phenanthroline, benzothiazole, benzoxazole, Stokes shift, lead ions, fluorescent sensors

Introduction

Fluorescent sensors are powerful tools, used in the *in vitro* and/or *in vivo* monitoring of biologically relevant species such as metal ions, because of the simplicity and high sensitivity of fluorescence spectroscopy.[1] A typical fluorescent sensor contains a recognition site, in this case the ionophore, linked to a fluorophore which translates the recognition event into a fluorescence signal.[2] The design of the "small molecule" probes described herein involves the selection of the phenanthroline moiety as ionophore, since this dinitrogen heterocyclic system coordinates efficiently with heavy atom ions.[3-7]

The selection of the fluorophore required more careful consideration: a large Stokes shift is a highly desired characteristic for a fluorescent probe since it permits an efficient separation of the light exciting the matrix and the light dispersed by the sample.[8] Excited-state intramolecular

proton transfer (ESIPT) is a reaction that can produce emissive species with sizeable, often by 100 nm or more, long wavelength shifts on fluorescence spectra. The ESIPT process generally involves a hydroxyl proton transfer to an acceptor in the excited state, resulting in tautomer emission with a large Stokes shift.[9] 2-(2-Hydroxyphenyl)benzazoles are handy fluorescent molecules which show high Stokes shifts owing to this mechanism (Scheme 1).



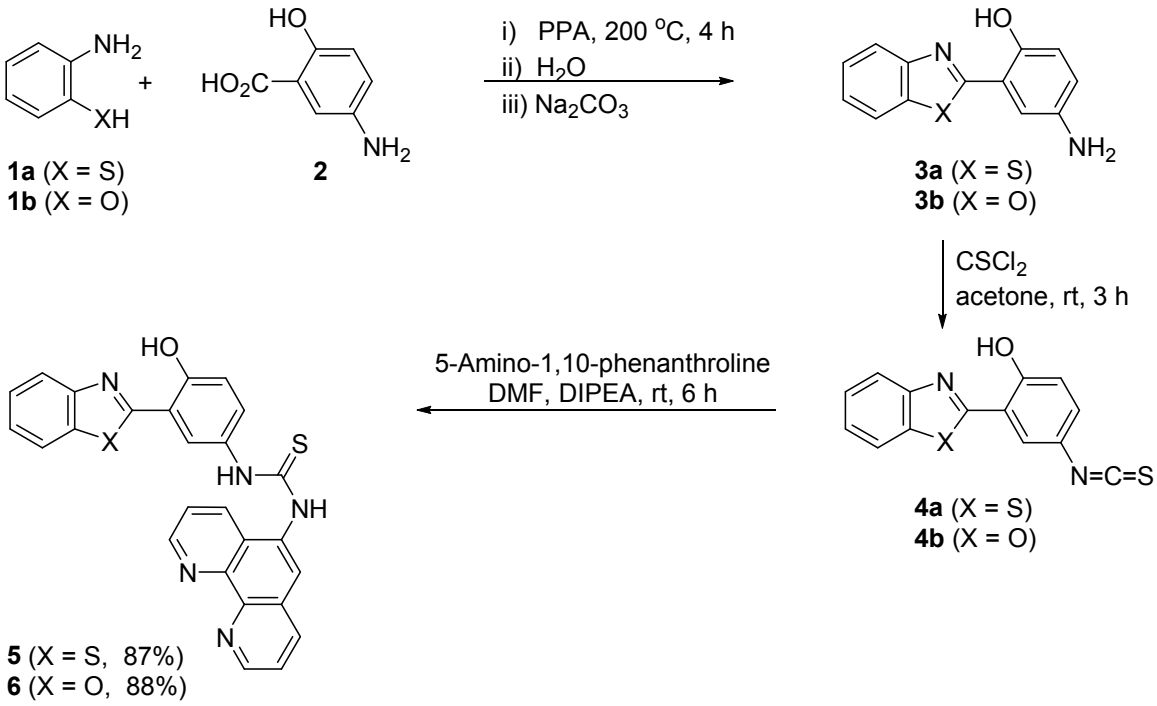
Scheme 1. Prototautomers involved in excited-state intramolecular proton transfer (ESIPT).

Results and Discussion

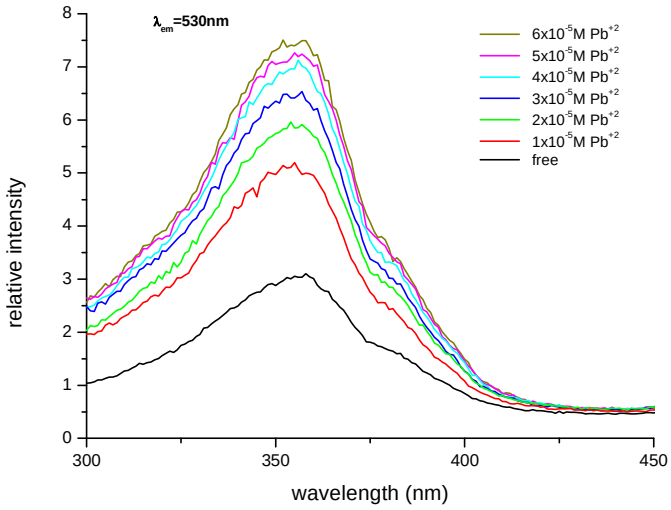
4-Amino-2-(benzothiazol-2-yl)phenol (**3a**) and 4-amino-2-(benzoxazol-2-yl)phenol (**3b**) were prepared by heating 5-aminosalicylic acid (**2**) with 2-aminothiophenol (**1a**) or 2-aminophenol (**1b**), respectively, in the presence of polyphosphoric acid as described in the literarure.[10-12] The amines were converted into the respective isothiocyanates **4a** and **4b** by treatment with excess thiophosgene in acetone at ambient temperature. Sensors **5** and **6** were prepared by mixing the isothiocyanates with 5-amino-1,10-phenanthroline in DMF in the presence of catalytic amounts of 4-diisopropylethylamine (Scheme 2).

Fluorescence studies on sensor **6** in the presence of increasing Pb^{2+} concentrations revealed the profile of a photoinduced electron transfer (PET) indicator with a distinct "turn on" response in low micromolar Pb^{2+} ion levels, with a large Stokes shift of 170 nm (Figure 1). Similar responses have been reported previously by our group[13] and others,[10] and are interpreted based on the analogous 2-(2-benzoxazolyl)- and 2-(2-benzothiazolyl)phenol systems that undergo an intramolecular proton transfer at the excited state (ESIPT). This is a very fast (picosecond) mechanism, faster than that of the electron transfer, and is dictated by a tautomeric equilibrium that yields the ESIPT product upon excitation.[14-19]

This effect is responsible for the observed high Stokes shift. Upon excitation of the ion-free probe **6** an electron is transferred from the phenanthroline moiety to the fluorophore and fluorescence is quenched. At micromolar concentrations, the Pb^{2+} ions coordinate with the nitrogen atoms of the phenanthroline system preventing the electron transfer, thus leading to a "turn on" response mode of the sensor **6** to the presence of Pb^{2+} ions. The quantum yield of the sensor **6** was calculated as $\Phi = 0.045$ that increased to $\Phi = 0.11$ at saturating Pb^{2+} ion concentrations.[20-22]



Scheme 2. Synthesis of fluorescent heavy metal sensors **5** and **6.**



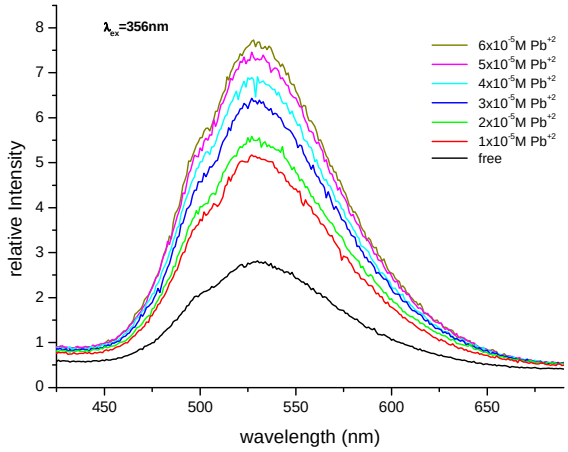
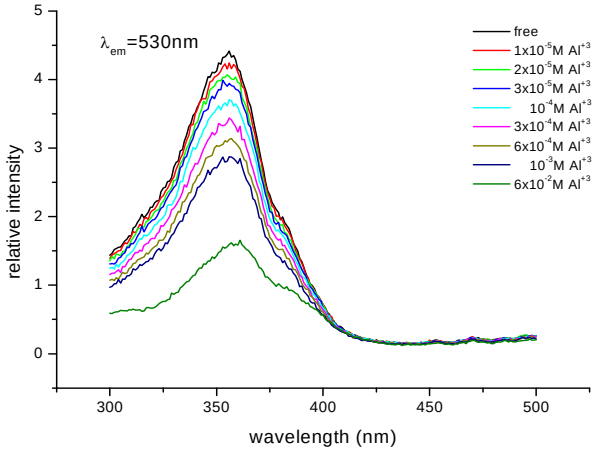


Figure 1. Excitation (top) and emission spectra (bottom) of sensor **6** (5 μM) in nanopure water, pH adjusted at 7.0, at millimolar Pb^{2+} ion concentrations. Excitation set at 356 nm, emission maxima detected at 530 nm.

Binding of sensor **6** to a host of metal ions including Cd^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+} and Mn^{2+} was registered by strong fluorescence quenching. This "turn off" spectral response is known for phenanthroline-type probes such as Phen Green FL and Phen Green SK.[4] The only exception was that of Al^{3+} interaction that resulted in a fluorescence decrease at 540 nm with a simultaneous increase at 450 nm and a clear isoemissive point at 480 nm. A possible explanation of this behavior is that the Al^{3+} ions would coordinate with the phenolic oxygen influencing the chromophore and therefore cancelling the ESIPT mechanism. Owing to this effect, a blue shift in the emission spectrum was observed, the Stokes shift decreasing from 185 to 95 nm. This effect is shown on Figure 2 depicting a ratiometric-type fluorescence response of the sensor **6** at high micromolar Al^{3+} ion concentrations.



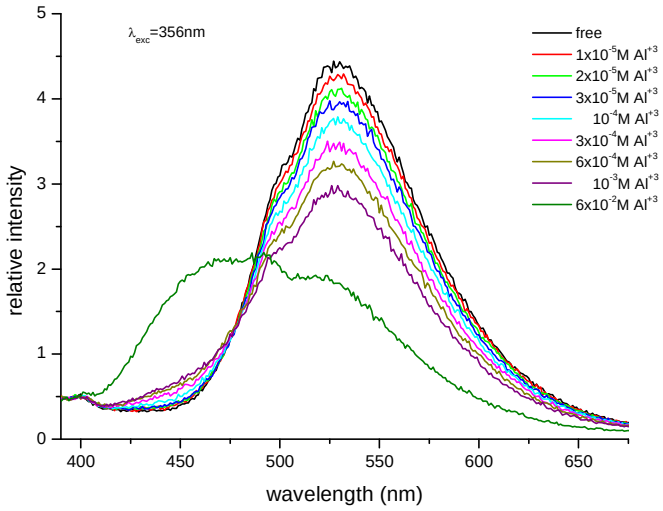


Figure 2. Excitation (top) and emission spectra (bottom) of the sensor **6** (5 μM) in nanopure water, pH adjusted at 7.0 at millimolar Al^{3+} ion concentrations. Excitation set at 356 nm, emission maxima detected at 530 nm.

Table 1. Spectral profile of sensors **5** and **6** in the presence of increasing ion concentrations

Sensor	**5**				**6**			
	λ_{exc} (nm)	λ_{em} (nm)	K_d (μM) [a]	Response	λ_{exc} (nm)	λ_{em} (nm)	K_d (μM) [a]	Response
Ion								
Pb^{2+}	360	554	nd[b]	Turn off	356	530	13.13	**Turn on**
Cd^{2+}	360	554	nd[b]	Turn off	356	530	nd[b]	Turn off
Cu^{2+}	360	554	nd[b]	Turn off	356	530	nd[b]	Turn off
Fe^{3+}	360	554	118.76	Turn off	356	530	24.65	Turn off
Fe^{2+}	360	554	nd[b]	Turn off	356	530	nd[b]	Turn off
Hg^{2+}	360	554	4.66	Turn off	356	530	nd[b]	Turn off
Mg^{2+}	360	554	7.12	Turn off	356	530	nd[b]	Turn off
Mn^{2+}	360	554	nd[b]	Turn off	356	530	nd[b]	Turn off
Co^{2+}	360	554	nd[b]	Turn off	356	530	nd[b]	Turn off
Ni^{2+}	360	554	nd[b]	Turn off	356	530	nd[b]	Turn off
Al^{3+}	360	554	nd[b]	Turn off	356	540/450	580.45	Ratiometric

[a] K_d values were detected in cases where the decrease in emission intensity of sensors **5** or **6** in the presence of increasing ion concentrations was distinct. [b] nd = not detected.

The fluorescence behavior of sensor **5** to a host of ions including Pb^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Al^{3+} is presented on Table 1. The response of the sensor was in all cases a "turn off" one, with dissociation constants in the micromolar range. The ion-free probe exhibits a λ_{exc} maximum of 360 nm with a respective λ_{em} at 554 nm and a quantum yield of $\Phi = 0.025$. Given the structural similarity of sensors **5** and **6**, the large (194 nm) Stokes shift of the former sensor is, as expected, the result of the ESIPT mechanism.

Metal-ion response screening for ion probes 5 and 6

A graphical overview of the relative responses of the two sensors to 30 μM metal ion solutions is depicted in Figure 3. The results are plotted as fluorescence changes relative to those of the ion-free reference solutions of sensors **5** or **6**, expressed as (F–Fo)/Fo, where F is the fluorescence intensity of ion-containing solutions and Fo is the fluorescence intensity of each reference solution. Blue bars indicate the response to 30 μM ion concentration.

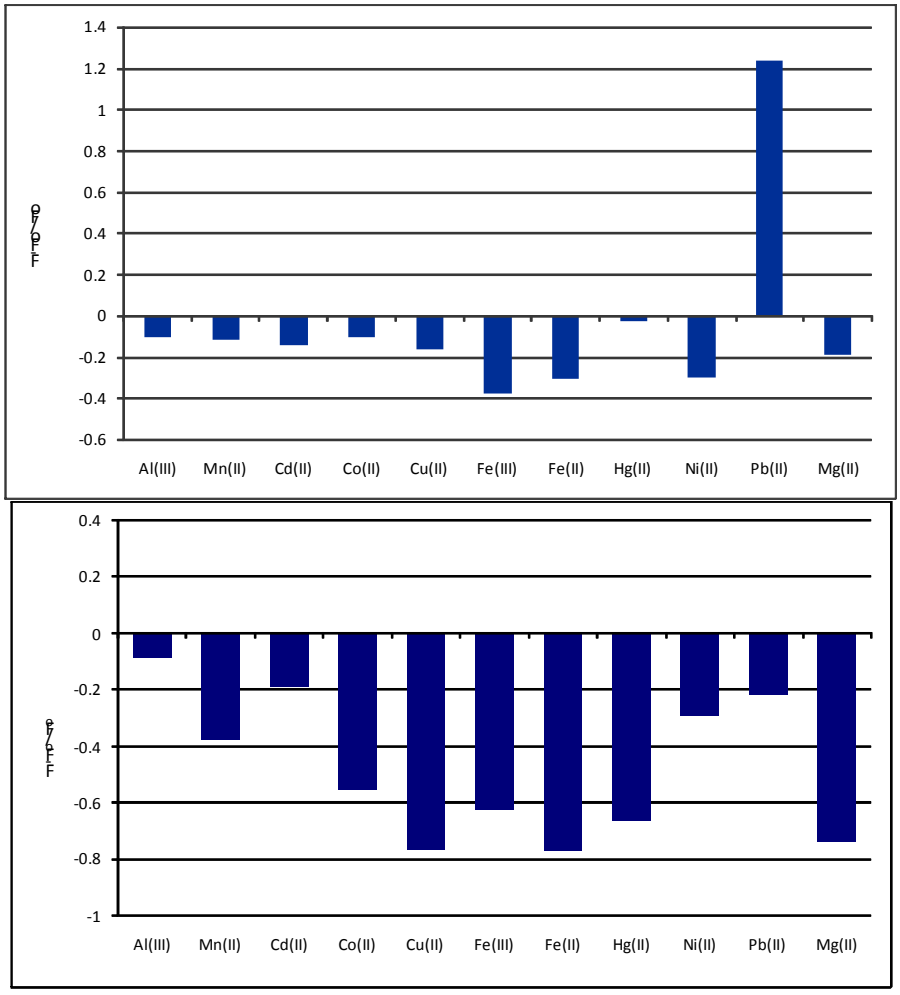
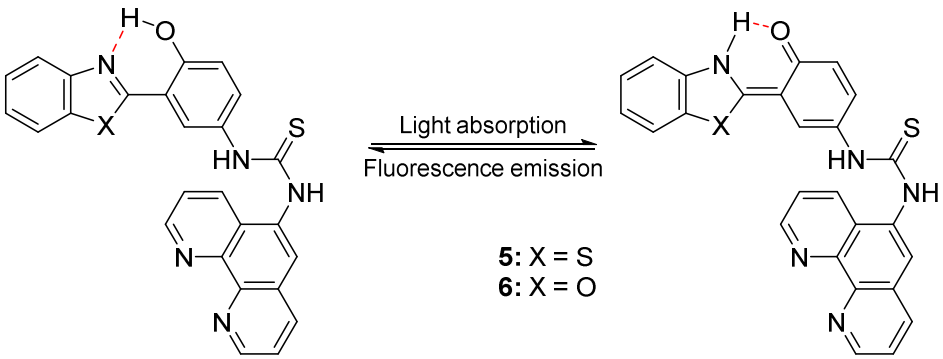


Figure 3. Fluorescence response of sensor **6** (top) and **5** (bottom) to 30 μM metal ion solutions. Results are shown as bar diagrams and are expressed as (F–Fo)/Fo, where F is the fluorescence

intensity of ion-containing solutions and Fo is the fluorescence intensity of each reference solution.

Worth noticing is the "turn-on" type of response of sensor **6** in the presence of Pb^{2+} ions as opposed to the rest of the ions studied, a behavior that is not observed in the case of sensor **5**. This difference in response should rest in the inherent properties of both sensors. Pb^{2+} is a "soft" metal ion and would favor a stronger coordination with the "soft" sulfur atom in the benzothiazole moiety in sensor **5**, whereas a weaker interaction of Pb^{2+} with the "hard" oxygen in sensor **6** is expected. It is therefore, possible that the stronger interaction of Pb^{2+} with the sulfur ion in sensor **5** would result in a decrease in binding with the phenanthroline nitrogens attenuating its interference with the electron transfer process. Moreover, the availability of either heteroatom is retained even upon excitation of the compounds as shown in Scheme 3. This selectivity towards the Pb^{2+} ions in phenanthroline probes where O-donor atoms are replaced by other softer donor atoms such as S and N is well known.[23,24]



Scheme 3. Prototautomers involved in excited-state intramolecular proton transfer (ESIPT) of compounds **5** or **6**. Structural changes that take place upon excitation do not alter the availability of heteroatom X for metal ion coordination.

Conclusions

Two new small-molecule sensors were prepared *via* short and efficient syntheses. The benzoxazole-phenanthroline type probe **6** exhibited a "turn-on" behavior selectively for Pb^{2+} ions and a ratiometric profile response to Al^{3+} ions; the latter response however, takes place at 45-fold higher concentrations. The response of probe **6** to the rest of metal ions studied is a "turn off" one with relatively smaller responses, thus rendering **6** a potential lead ion sensor in aqueous solutions. The benzothiazole congener **5** was a "turn off" probe responding to the presence of metal ions with up to 80% decrease in fluorescence intensity, with Cu^{2+}, Fe^{3+}, Fe^{2+}, Hg^{2+}, and Mg^{2+} inducing the largest responses. All studies were performed in aqueous solutions, a fact

implying that both sensors may be practical tools in analytical measurements and the detection of ions in biological studies.

Experimental Section

General. All reactions were carried out under anhydrous conditions in dry solvents, using argon or nitrogen in flame-dried glassware. Reactions were monitored by thin-layer chromatography (TLC) using silica gel plates from Merck ($60F_{254}$), which were visualized under a UV-vis Lamp (254 and 366 nm, respectively) or with a 7% ethanolic solution of phosphomolybdic acid. Flash column chromatography was performed in silica gel 60 from Merck (230-400 mesh). The Attenuated Total Reflection (ATR)-FTIR spectra were recorded on a Thermo-Electron Nicolet 6700 FTIR optical spectrometer with a DTGS KBr detector at a resolution of 4 cm^{-1}. NMR spectra were taken on an AMX500 Bruker FT-NMR or a MSL300 Bruker FT-NMR spectrometer; proton chemical shifts are reported in relative to tetramethylsilane. Fluorescence spectra were recorded on an Aminco Bowman spectrofluorimeter (Spectronics Co., USA). HRMS were taken at ProFI, Foundation for Research and Technology-Hellas (ITE), Heraklion, Greece. Ultra-pure water was collected from a PURELAB Ultra instrument by ELGA.

4-Amino-2-(benzothiazol-2-yl)phenol (3a) and 4-amino-2-(benzoxazol-2-yl)phenol (3b). Prepared by heating 5-aminosalicylic acid (**2**) with 2-aminothiophenol (**1a**) or 2-aminophenol (**1b**), respectively, in the presence of polyphosphoric acid as described in the literarure.[10-12]

2-(1,3-Benzothiazol-2-yl)-4-isothiocyanatophenol (4a). To a solution of the aminophenol **3a** (245 mg, 1.01 mmol) in acetone (10 mL) was added dropwise over a 10 min period a 5-fold excess of thiophosgene (0.390 mL, 5.05 mmol). The mixture was stirred under argon atmosphere for 2 h and then the volatiles were removed *in vacuo*. [**Caution:** removal of unreacted thiophosgene should take place with great care owing to the toxicity of the reagent.] The yellowish solid product was suspended in cold acetone, filtered and dried under vacuum to afford the *title compound* **4a** (280 mg, 97%) as a green-grey powder, mp 158-159 ^{o}C (not recrystallised). FTIR (cm^{-1}): (N=C=S) 2108. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 9.91 (s, 1H), 8.31 (s, 1H), 8.14 (d, *J* 8.4 Hz, 1H), 8.10 (br.s, 1H), 8.03 (br.s, 1H), 7.51-7.45 (m, 1H), 7.44-7.30 (m, 1H), 7.22-7.08 (m, 1H). ^{13}C NMR (500 MHz, $CDCl_3$) δ_C 162.4, 155.6, 151.3, 135.1, 133.0, 129.5, 126.7, 125.6, 125.4, 122.5, 122.2, 121.8, 119.9, 118.4. HRMS (ESI) calc for $C_{14}H_9N_2OS_2$ *m/z* (MH^+) 285.0150, obsd 285.0181.

2-(1,3-Benzoxazol-2-yl)-4-isothiocyanatophenol (4b). Prepared following the same procedure described for **4a** starting with aminophenol **3b** (226 mg, 1.0 mmol) and thiophosgene (0.390 mL, 5.05 mmol) in acetone (10 mL). Yields of the *title compound* **4b** ranged from 249 to 255 mg (93-95%) of a yellow powder, mp 159-161 ^{o}C (not recrystallised). FTIR (cm^{-1}): (N=C=S) 2114. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 11.61 (br.s, 1H, D_2O exchangeable), 7.90 (d, *J* 2.5 Hz, 1H), 7.77-7.75 (m, 1H), 7.66-7.64 (m, 1H), 7.45, (dd, *J* 7.5, 5.5 Hz, 1H), 7.42 (dd, *J* 7.5, 5.5 Hz, 1H), 7.31

(dd, *J* 8.5, 2.5 Hz 1H), 7.10 (d, *J* 8.5 Hz, 1H). ^{13}C NMR (500 MHz, $CDCl_3$) δ_C 161.3, 157.5, 149.1, 148.7, 139.6, 130.6, 126.0, 125.4, 124.0, 123.0, 119.5, 118.8, 111.6, 110.9. HRMS (ESI) calc for $C_{14}H_9N_2O_2S$ *m/z* (MH^+) 269.0379, obsd 269.0407.

***N*-[3-(1,3-Benzothiazol-2-yl)-4-hydroxyphenyl]-*N'*-[1,10]phenanthrolin-5-yl-thiourea (5).** In a flame-dried 50 mL round bottomed flask were placed DMF (8 mL) and the isocyanate **4a** (142.0 mg, 5.0 mmol) followed by 5-amino-1,10-phenanthroline (97.0 mg, 0.5 mmol). The mixture was then stirred in the dark, under an argon atmosphere for 2 h. The solution was then diluted with water (10 mL) and the pH was adjusted to 7.5 by dropwise addition of 1N HCl solution. The precipitate (207 mg) was filtered and washed with water (15 mL) then with Et_2O (15 mL). The crude product was repeatedly suspended in Et_2O and then centrifuged to remove traces of side products and yielded the *title compound* **5** (94 mg, 40%) as a green grey powder, mp 164-165 ^oC (not recrystallised). FTIR (cm^{-1}): (C=S) 1574. ^{1}H NMR (500 MHz, DMSO-d_6) 10.89 (br.s, 1H), 9.26 (d, *J* 4.0 Hz, 1H), 9.20 (d, *J* 4.0 Hz, 1H), 9.04 (dd, *J* 10.5, 9.0 Hz, 2H), 8.85 (br.s, 2H), 8.47 (s, 1H), 8.35 (d, *J* 2.0 Hz, 1H), 8.19-8.15 (m, 2H), 8.09 (d, *J* 8.0 Hz, 1H), 8.01 (d, *J* 8.0 Hz, 1H), 7.59 (dd, *J* 8.5, 2.0 Hz, 1H), 7.49 (dd, *J* 7.5, 7.5 Hz, 1H), 7,39 (dd, *J* 7.5, 7.0 Hz, 1H), 7.13 (d, *J* 8.5 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ_C 181.9, 164.7, 153.8, 151.4, 148.6, 146.5, 141.9, 138.9, 136.7, 136.6, 135.2, 134.6, 131.2, 129.3, 129.2, 127.7, 126.4, 125.5, 125.2, 125.00, 124.1, 123.5, 122.1, 122.00, 118.2, 116.9. HRMS (ESI) calc for $C_{26}H_{18}N_5OS_2$ *m/z* (MH^+) 480.0947, obsd 480.0909.

***N*-[3-(1,3-Benzoxazol-2-yl)-4-hydroxyphenyl]-*N'*-[1,10]phenanthrolin-5-yl-thiourea (6).** To a solution of the isocyanate **4b** (134 mg, 0.5 mmol) in dry DMF (8 mL), was added 5-amino-1,10-phenanthroline (97.0 mg, 0.5 mmol). The system was stirred in the dark, under an argon atmosphere for 2 h. After the completion of the reaction, as indicated by TLC (50% EtOAc in petroleum ether), the solution was then diluted with water (10 mL) and the pH adjusted to 7.5 by dropwise addition of 1N HCl solution. The precipitate was filtered and washed with water (15 mL) then with Et_2O (15 mL). The crude product (204 mg) was repeatedly suspended in Et_2O and then centrifuged to remove traces of side products to afford the *title compound* **6** (95 mg, 41%) as a yellow powder, mp 169-170 ^oC (not recrystallised) slightly soluble in DMSO at high dilutions. FTIR (cm^{-1}): (C=S) 1522. ^{1}H NMR (500 MHz, DMSO-d_6) 9.90 (br.s, 1H), 9.18 (dd, *J* 15.0, 3.5 Hz, 1H), 9.02 (br.d, *J* 16.5 Hz, 1H), 8.84 (dd, *J* 16.5, 7.0 Hz, 1H), 8.72 (dd, *J* 7.5, 7.5 Hz, 1H), 8.52 (br.d, *J* 11.5 Hz, 1H) 8.47-8.42 (m, 1H), 8.22-8.11 (m, 1H), 8.05-7.84 (m, 3H), 7.35-7.22 (m, 1H), 7.33 (d, *J* 7.5 Hz, 1H). HRMS (ESI) calc for $C_{26}H_{18}N_5O_2S$ *m/z* (MH^+) 464.1175, obsd 464.1219.

Preparation of indicator solutions containing adjusted ion concentrations

In 3 mL nanopure water (pH = 7) were added 15 μL aliquots of a 10 mM in DMSO (≥99.5%) dye solution to make a final indicator concentration of 5 μM. To this solution were added microliter aliquots of metal ion stock 10^{-2} M solutions to yield a set of broad ion concentration range dye solutions.

Acknowledgements

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