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PII: S0040-4020(17)30630-0

DOI: 10.1016/j.tet.2017.06.011

Reference: TET 28776

To appear in: *Tetrahedron*

Received Date: 8 April 2017

Revised Date: 1 June 2017

Accepted Date: 2 June 2017

Please cite this article as: Chen W, Zhu L, Hao Y, Yue X, Gai J, Xiao Q, Huang S, Sheng J, Song X, Detection of thiophenol in buffer, in serum, on filter paper strip, and in living cells using a red-emitting amino phenothiazine boranil based fluorescent probe with a large Stokes shift, *Tetrahedron* (2017), doi: 10.1016/j.tet.2017.06.011.

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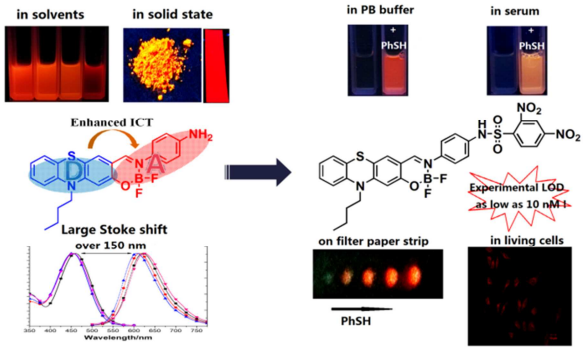

Detection of thiophenol in buffer, in serum, on filter paper strip, and in living cells using a red-emitting amino phenothiazine boranil based fluorescent probe with a large Stokes shift

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Abstract

A novel red-emitting dye, **PB-NH_2**, was synthesized by incorporation of an electron rich phenothiazine moiety to classical boranil dye. **PB-NH_2** displayed excellent photophysical properties, such as long emission wavelength, large Stokes shift, strong emission both in solution and in solid state. Based on this attractive platform, **Probe 1**, was constructed for selective detection of thiophenol. Notably, **Probe 1** was ultrasensitive in response to thiophenol, and the corresponding detection limits for thiophenol in theoretical and in experimental were determined to be 1.4 and 10 nM, respectively. Significantly, **Probe 1** showed great potential for practical applications, and the applications in real water samples, in serum, on filter paper strips and in living cells were successfully demonstrated.

Key words: phenothiazine boranil; fluorescent probe; red-emitting; large Stokes shift; thiophenol detection

1. Introduction

Thiophenols have broad synthetic utility and they are widely used in chemical industry, such as the preparation of pesticides, medicine and various industrial products.[1-3] However, despite their great practical value, thiophenols have been listed as one of the prioritized pollutants by the United States Environmental Protection Agency (USEPA) due to the highly toxic property.[4] Long-term exposure of thiophenols liquid or vapor is extremely detrimental to human health, which may induce severe central nervous system damage and other related system injuries, even death.[5] Toxicological studies revealed that thiophenols process the median lethal concentration (LC50) values of 0.01-0.4 mM in fish and a median lethal dose (LD50) value of 46.2 mg/kg in mouse.[6-7] Therefore, the development of highly sensitive and selective methodologies for the detection of thiophenols is of considerable significance in the fields of chemical, biological and environmental science. Owing to the advantages of simplicity and high sensitivity as well as non-invasiveness and high spatiotemporal resolution, fluorescent methods have been recognized as the effective tools that can help monitor and visualize various cations, anions, and biomolecules both *in vitro* and *in vivo*.[8] In the past decade, great efforts have been devoted to the development of fluorescent probe for thiophenols over aliphatic thiols. Pioneered by Wang's group, the selective detection of thiophenols was realized by taking advantage of the thiophenols-mediated cleavage of sulphonamide.[9] From then on, a variety of fluorescent probes capable of detecting thiophenols selectively had been reported based on the extended version of the above-mentioned strategy.[10-34] Despite the significant advances have obtained so far, it is noteworthy that most of the reported probes suffer from short emission wavelength and relatively small Stokes shift. It is well known that fluorescent probes with emission in red or near-infrared range are optimal for biological imaging applications due to the deep tissue penetration, decreased light scattering, reduced autofluorescence and minimum

photodamage.[35] Moreover, fluorophores with large Stokes shift are quite suitable for fluorescence microscopy studies, because the large Stokes shift enables the clearly separated excitation and emission bands, which can effectively minimize the interferences caused by self-absorption or auto-fluorescence.[10] Unfortunately, red or near-infrared fluorescent probes for thiophenol with large Stokes shift are rare up until now. Among the reported thiophenol probes, three fluorescent probes emit in red or near-infrared region ($\lambda_{em} \geq 600$ nm) as well as display large Stokes shift (≥ 120 nm); they could detect thiophenol in solutions, but not suitable for thiophenol detection in solid state due to the severe ACQ (aggregation-caused quenching) process (see Table S1). Fluorophores that exhibit large Stokes shift and strong red or near-infrared fluorescence both in solution and in solid state are highly desirable for thiophenol probes development, from the stand point of practical application.

In this work, we have exploited a unique amino phenothiazine boranil dye **PB-NH$_2$**, which displays strong red emission not only in solutions but also in solid state. More importantly, the Stokes shift of **PB-NH$_2$** could reach to incredibly 172 nm in EtOH/PBS buffer (v/v=1:1) solution. Thereby, **PB-NH$_2$** offers a robust platform upon which novel fluorescent probe for thiophenol was based. Herein, we synthesized a novel fluorescent **Probe 1** for thiophenol by incorporation of a dinitrobenzene sulfonate (DNBS) moiety to **PB-NH$_2$**, which could detect thiophenol both in aqueous solutions and in solid state.

2. Results and discussion

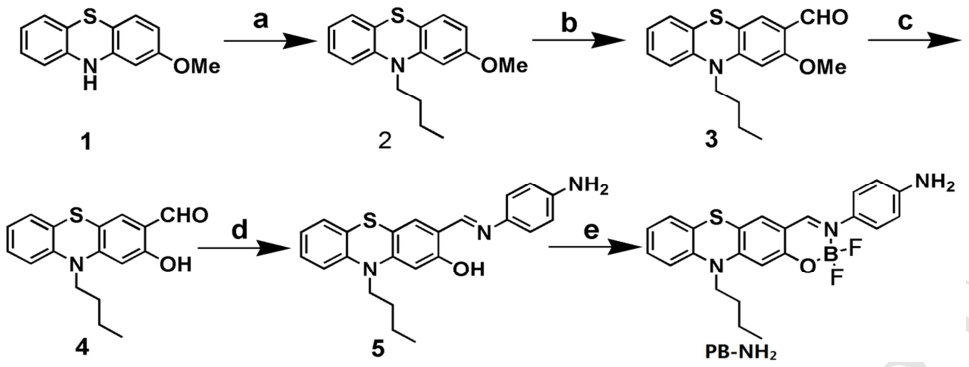
3.1 Design rational

In recent years, heteroatom-modified organic dyes, such as BODIPYs have received considerable attention due to their outstanding optical characteristics, such as relatively narrow absorption and emission band, high molar absorption coefficient, intense fluorescence quantum yield, satisfactory photostability and chemostability, and exceptional insensitivity to the polarity of solvents and to pH.[36] However, classical BODIPYs suffered from severe fluorescence

quenching in solid state due to the tight intermolecular π-π stacking. Moreover, most BODIPYs exhibit very small Stokes shift (< 20 nm, in most cases), which inevitably results in self-quenching and in measurement errors by excitation and scattering light.[41] Therefore, the development of novel red emissive boron difluoride complexes with large Stokes shift is of great importance. In fact, this also represents the current state-of-art in the field of novel BODIPY analogues exploitation.

Phenothiazine, which was first synthesized by Bernthsen in 1883,[38] has been widely used in a variety of industries including the manufacturing of dyes and pigments, drugs, dye-sensitized solar cells and photocopying materials.[39] Phenothiazine contains electron-rich nitrogen and sulfur heteroatoms in a heterocyclic structure with strong electron-donating ability. We anticipate that the introduction of a phenothiazine moiety to BODIPY analogues may serve as a robust strategy for developing red-emitting fluorescent dyes with large Stokes shift. Moreover, the nonplanar butterfly-like molecular conformation of phenothiazine can effectively preclude the intermolecular π-π stacking,[40] thereby leading to strong solid fluorescence.

As a proof-of concept, we designed novel amino phenothiazine boranil dye, **PB-NH$_2$**, by incorporation of a phenothazine moiety to classical amino boranil dye, and readily synthesized it from commercial available compound **1** in five steps (Scheme 1). Condensation of compound **1** with n-bromobutane to form compound **2**, the subsequent Vilsmeier acylation with $POCl_3$/DMF obtained phenothiazine-2-methoxy-3-aldehyde (compound **3**). Demethylation of compound **3** by AlI_3 (prepared in suit) afforded the key intermediate phenothiazine salicylaldehyde **4**, which was then treated with *p*-phenylenediamine formed Schiff base **5**. Finally, the BF_2 complexation of compound **5** and $BF_3 \cdot Et_2O$ in 1,2-dichloroethane generated the target dye **PB-NH$_2$**. The chemical structures of all compounds were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS (see supporting information).



Scheme 1. Synthetic route of **PB-NH$_2$**. Reagents and conditions are as follows: (a) n-bromobutane, NaOH, KI, DMSO, 100°C, Ar, 6 h; (b) $POCl_3$/DMF, 0°C, Ar, 15 min, then compound 2, DMF, 60°C, 4 h; (c) Aluminium powder, iodine, CH_3CN, room temperature, Ar, 30 min, then compound 3, CH_3CN, 80°C, 6 h; (d) p-phenylenediamine, ethanol, room temperature. (e) $BF_3 \cdot Et_2O$, DIEA, 1, 2-dichloroethane, reflux, 5 h.

With dye **PB-NH$_2$** in hand, we were eager to investigate its optical properties in different solvents. The absorption and emission spectra of **PB-NH$_2$** in different solvents (dichloromethane, EtOH, pH 7.4 PBS buffer/EtOH (v/v=1:1) and CH_3CN) are shown in Figure S1, and the corresponding photophysical data are summarized in Table 1. As designed, **PB-NH$_2$** displays emission peaks well into the red region, with maximum at 605-626 in different solvents. Moreover, **PB-NH$_2$** has the fluorescence quantum yields of 0.08-0.28 in different solvents, which are great values for red-emitting dyes with large Stokes shift. Remarkably, **PB-NH$_2$** exhibits large Stokes shifts of 154-172 nm in different solvents, which are much superior to those of classical BODIPYs (< 20 nm in most cases). In addition, **PB-NH$_2$** is a new solid-emissive fluorophore, which shows strong red fluorescence in powder state and on test paper strip (Figure 1)

Table 1. Photophysical data of **PB-NH$_2$** in different solvents

Solvent	λ_{abs}/nm[a]	λ_{em}/nm[b]	Stokes shift	Φ_f[c]	τ[d]/ns
dichloromethane	460	621	161	0.28	3.68
CH_3CN	453	613	160	0.20	2.81
EtOH	451	605	154	0.19	2.62
EtOH/PBS (1:1)	454	626	172	0.08	1.16

[a]The maximal absorption of the **PB-NH$_2$**. [b]The maximal emission of the **PB-NH$_2$**. [c]Φ_f is the

relative fluorescence quantum yield estimated by using fluorescein (Φ_f = 0.79 in ethanol) as a standard. [d]Fluorescence lifetime.

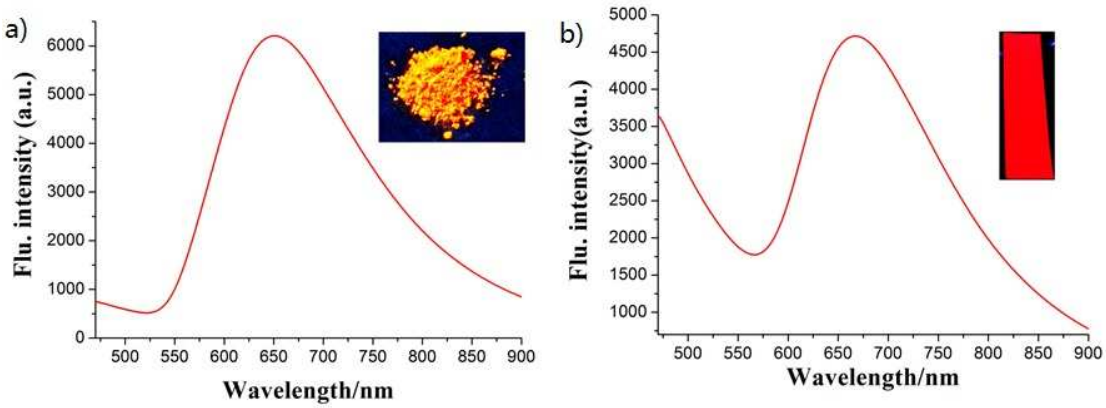
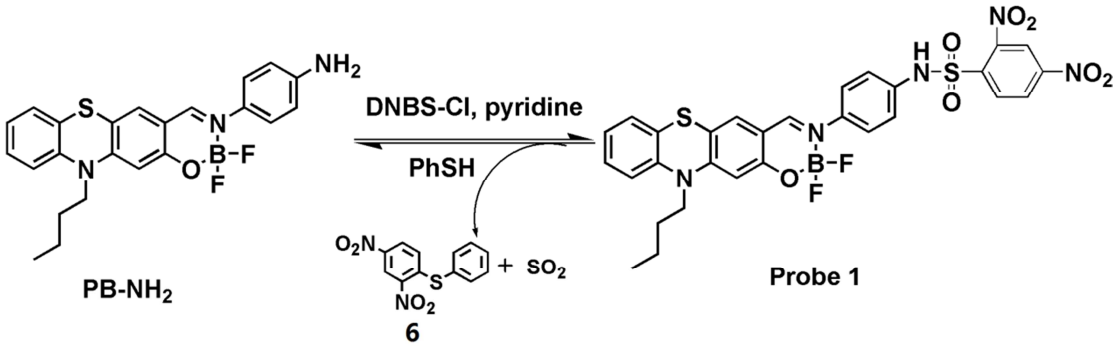


Figure 1. (a) Fluorescence spectrum of PB-NH_2 powder. Insert: photograph of PB-NH_2 powder under a UV 365 lamp; (b) Fluorescence spectrum of PB-NH_2 coated filter paper strip. Insert: photograph of PB-NH_2 coated filter paper strip under a UV 365 lamp.

Inspired by these encouragine findings, we anticipate that **PB-NH_2** could be employed as an attractive platform upon which a novel fluorescent probe, that allows thiophenol detection both in aqueous solutions and in solid state, is based. With all these in mind, we designed and synthesized **Probe 1** by incorporation of a DNBS moiety to **PB-NH_2** (Scheme 2) The chemical structure of **Probe 1** was fully confirmed by ^{1}H NMR, ^{13}C NMR and HRMS (see supporting information). The design rationale is also depicted in Scheme 2 and illustrated as follows. The DNBS moiety was selected because it functions not only as a recognition group for thiophenol, but also as an effective fluorescence quencher via the photo-induced electron transfer (PET) process to ensure a low fluorescence background. Upon addition of thiophenol, the masked DNBS moiety can be removed smoothly through the S_NAr process, to release dye **PB-NH_2**, thereby resulting in a strong, time-dependent red fluorescence signal.



Scheme 2. Synthesis of **Probe 1** and the proposed response mechanism of **Probe 1** to thiophenol

3.2 Sensing properties of **Probe 1** to thiophenol

The sensing ability of **Probe 1** in response to thiophenol was investigated in PBS buffer (10 mM, pH = 7.4, containing 1 mM CTAB). As shown in Figure S2, free **Probe 1** displayed an absorption band centered at 465 nm. However, upon addition of the incremental amounts of thiophenol to the solution of **Probe 1**, the absorption peak at 465 nm was shifted to 445 nm and concomitant appearance of a new peak at 340 nm. The absorption peaks at 445 nm and 340 nm could be assigned to **PB-NH$_2$** and compound **6**, respectively, which were supported by the corresponding absorption comparison in Figure S3. Moreover, free **Probe 1** was essentially non-fluorescent, addition of thiophenol to the solution of **Probe 1** induced significant fluorescence enhancement at 621 nm (Figure S4). Importantly, **Probe 1** displayed a large Stokes shift of about 158 nm in response to thiophenol which can effectively reduce the interference from self-absorption and therefore can greatly improve the sensitivity in the fluorescence detection. To gain insight into the sensitivity of **Probe 1** in PBS buffer (10 mM, pH = 7.4, containing 1 mM CTAB), the fluorescence intensity of **Probe 1** in response to the incremental amounts of thiophenol was investigated. As expected, the fluorescence intensity increased gradually with incremental addition of thiophenol. When 1.4 equivalent of thiophenol was added to the solution of **Probe 1**, the fluorescence intensity reached a plateau and the enhancement was up to 104-fold (Figure 2). In addition, further study suggested a plot of fluorescence intensity at 621 nm versus the concentrations

of thiophenol exhibited good linearity (R = 0.9949) in the range of 0-12 μM. In addition, the theoretical detection limit was calculated to be 1.4 nM (based on S/N = 3) (Figure S5). Notably, the experimental detection limit is 10 nM, which displays an approximately 1.2-fold increase of the fluorescence intensity with good reproducibility (Figure S6). To the best of our knowledge, it was the lowest experimental detection limit ever reported. All these traits suggested that **Probe 1** was indeed a sensitive detector for quantitative analysis of thiophenol in aqueous media.

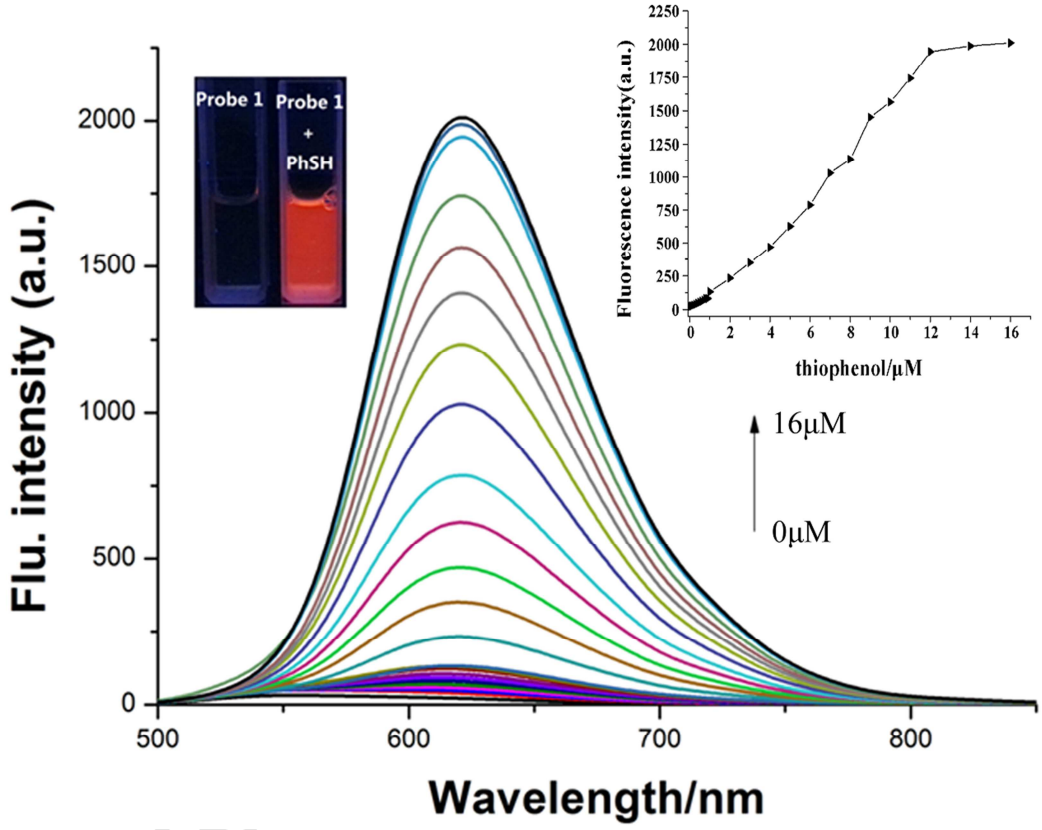


Figure 2. Fluorescence spectra of **Probe 1** (10 μM) in the presence of thiophenol (0-16 μM) in PBS buffer (10 mM, pH=7.4, containing 1 mM CTAB). Excited at 470 nm.

3.3 Kinetic studies

Time-dependent fluorescence arising from the interaction of aqueous **Probe 1** (10 μM) with 0.5 and 1.0 equivalent of thiophenol were investigated by monitoring the fluorescence intensity changes at 621 nm as a function of time. As shown in Figure 3, upon addition of thiophenol to the solution of **Probe 1**,

the fluorescence intensity at 621 nm increased immediately and reached a stable signal plateau within 5 min, indicating that **Probe 1** could respond to thiophenol rapidly. Moreover, this reaction obeys a typical pseudo-first-order with rate constant k_{obs} determined to be 0.7727 min^{-1} (Figure S7). In addition, in the absence of thiophenol, the solution of **Probe 1** displayed negligible changes in the fluorescence intensity at 621 nm during the identical interval suggesting that free **Probe 1** was highly stable in aqueous solution.

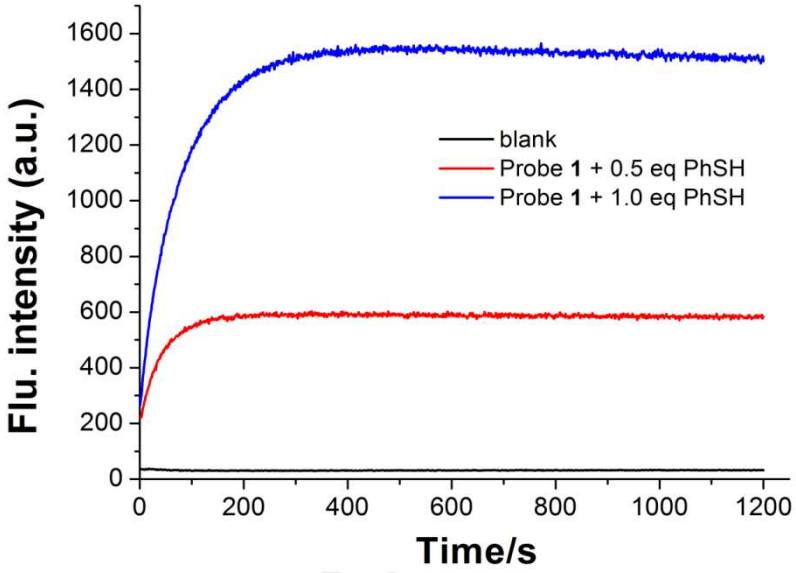


Figure 3. Kinetics of fluorescence enhancement rate at 621 nm for **Probe 1** (10 μM) in the absence (black line) and presence of 5 μM (red line) and 10 μM (blue line) thiophenol in PBS buffer (10 mM, pH=7.4, containing 1 mM CTAB).

3.4 Mechanism studies

The optical changes of **Probe 1** in response to thiophenol indicated the releasing of **PB-NH_2**. The proposed sensing mechanism is shown in Scheme 2. To further confirm the sensing mechanism, ^{1}H NMR titration was conducted for a mixture of **Probe 1** and thiophenol in DMSO-d_6. As shown in Figure 4, With the addition of incremental amounts of thiophenol to **Probe 1**, the signal peaks of **Probe 1**, especially those marked with (■), disappeared gradually, along with the concomitant appearance the signal peaks of **PB-NH_2** (peaks marked with (*), indicating the cleavage of 2,4-dinitrophenolate moiety in

Probe 1 and leading to the release of **PB-NH$_2$**. It is worthy to note that no obvious signal peak that assigned to the protons of amino group in **PB-NH$_2$** (peak marked with (★)) was observed after addition of thiophenol to the solution of **Probe 1**. We reasoned that this phenomenon may explained by the substitution of hydrogen atoms on amino group by deuterium atoms in DMSO-d_6. Moreover, all signal hallmarks of the reference compound **6** (peaks marked with (▼)) can be observed in the mixture of **Probe 1** and thiophenol, indicating the formation of compound **6** during the reaction process. Similarly, the HRMS spectrum of the reaction mixture of **Probe 1** and thiophenol displayed a main peak at m/z=460.1439 (Figure S8) which was nearly identical to the exact molecular weight of **PB-NH$_2$** ([M+Na$^+$]=460.1437). Additionally, HPLC analysis for **Probe 1**, **PB-NH$_2$** and the reaction mixture of **Probe 1** with thiophenol in PBS buffer (10 mM, pH 7.4, containing 1 mM CTAB and 5% DMF) was carried out (Figure S9). **Probe 1** displayed a single peak with a retention time at 2.88 min. Upon addition of 1.0 equiv. of thiophenol, the peak of **Probe 1** decreased dramatically and a new peak was emerged with a retention time 2.24 min, which was assigned to **PB-NH$_2$**. Taking together, all these results provide strong support for the proposed sensing mechanism in Scheme 2.

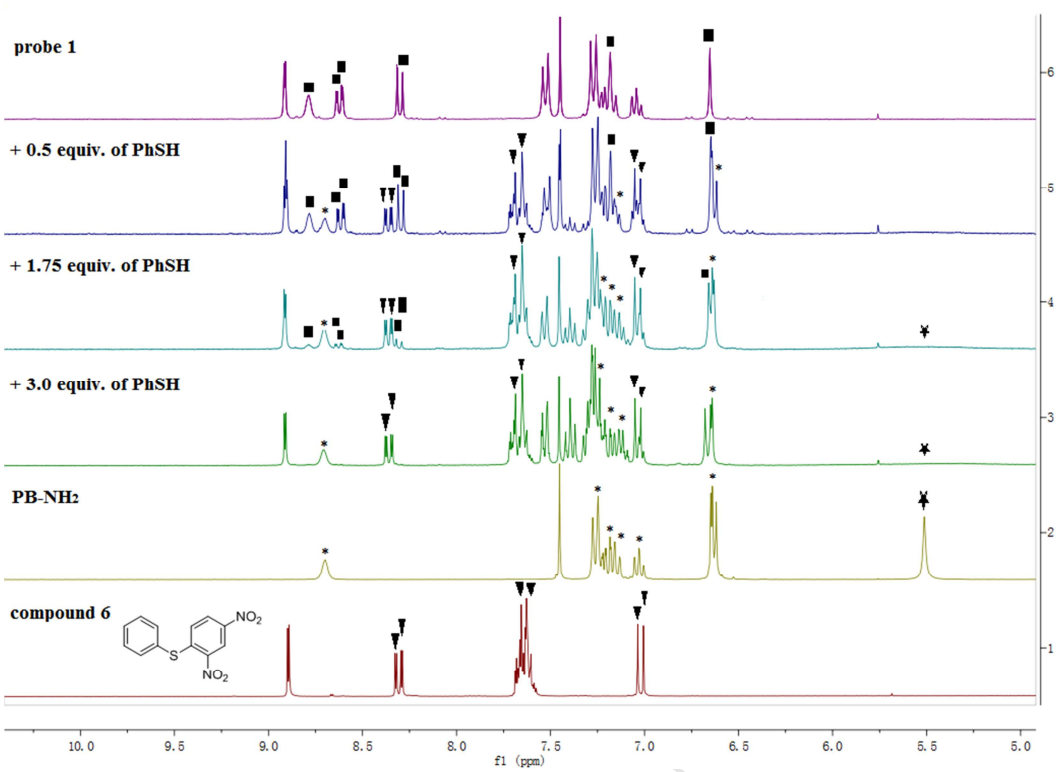


Figure 4. ^{1}H NMR spectral comparison of **Probe 1**, **Probe 1** with incremental amounts of thiophenol, **PB-NH$_2$** and compound **6**. All spectra were obtained in DMSO-d_6.

3.5 Selectivity and effect of pH

Another important parameter to evaluate the performance of a fluorescent probe is the selectivity. To gain insight into the selectivity of **Probe 1** toward thiophenol, the fluorescence behaviours of **Probe 1** in response to various potential interfering species, including aliphatic thiols (2-aminoethanethiol, DTT, Cys and Hcy, 0.1 mM for each, and 1 mM for GSH), some essential amino acids (Lys, Gly, Gln, Phe, Met, Tyr, Thr, Ser, His, Ile, Pro, Ala, Asp Arg and Val, 0.1 mM for each), representative anions (CH_3COO^-, $S_2O_3^{2-}$, HSO_3^-, SO_4^{2-}, PO_4^{3-},NO_2^-, NO_3^-, and S^{2-}, 0.1 mM for each) and biologically abundant metal ions (K^+, Ca^{2+}, Mg^{2+} and Zn^{2+}, 0.1 mM for each) as well as reactive oxygen species (H_2O_2 and ClO^-, 0.1 mM for each) were investigated. The result showed in Figure 5 only thiophenol induced a drastic fluorescence enhancement, no significant fluorescence changes were observed upon addition of other analytes, including the aliphatic thiols (1-mercaptopane, cysteamine,

DTT, Cys, Hcy and GSH). The drastic differences in fluorescence spectra allowed the naked eye differentiation of thiophenol from other analytes under a UV 365 nm lamp irradiation (Figure 5). Moreover, competition experiments were carried out by adding thiophenol (16 μM) to the solution of **Probe 1** (10 μM) in the presence of 100 μM of other interfering species. As shown in Figure S10, strong fluorescence was observed when **Probe 1** was exposed to thiophenol, which was not disturbed by other interfering species. Therefore, it can be concluded that **Probe 1** exhibits high selectivity toward thiophenol.

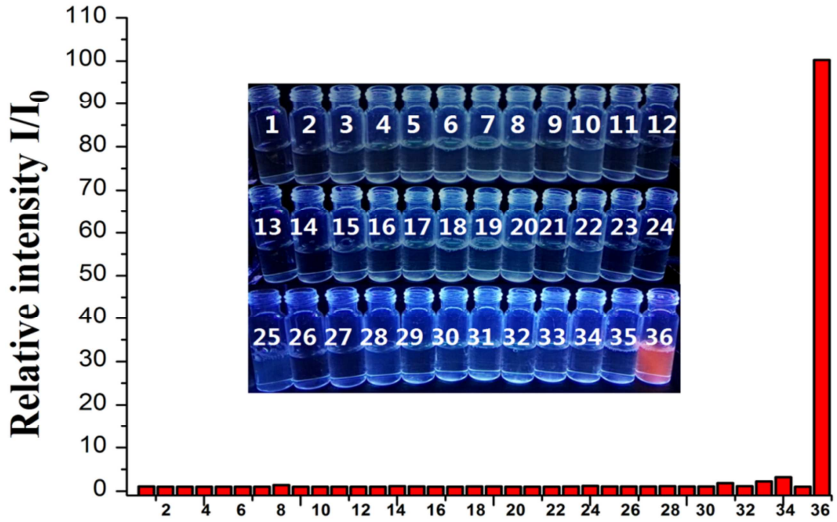


Figure 5. Selectivity of **Probe 1** (10 μM) toward thiophenol and other analytes in PBS buffer (10 mM, pH=7.4, containing 1 mM CTAB) for 30 min. Legend: (1) PO_4^{3-}, (2) CH_3COO^-, (3) $S_2O_3^{2-}$, (4) HSO_3^-, (5) SO_4^{2-}, (6) NO_2^-, (7) NO_3^-, (8) S^{2-}, (9) K^+, (10) Ca^{2+}, (11) Mg^{2+}, (12) Zn^{2+}, (13) H_2O_2, (14)ClO^-, (15) Lys, (16) Gly, (17) Gln, (18) Phe, (19) Met, (20) Tyr, (21) Thr, (22) Ser, (23) His (24) Ile, (25) Pro, (26) Ala, (27) Asp, (28) Arg, (29) Val, (30) Cys, (31) Hcy, (32) 2-aminoethanethiol, (33) DTT, 0.1 mM for each, (34) GSH, 1mM, (35) blank and (36) PhSH, 16 μM. Insert: Emission images of **Probe 1** with different analytes under UV 365 lamp.

To evaluate the limitations of **Probe 1** in aqueous solution, the effect of pH on the its fluorescence intensity in the absence and presence of thiophenol was investigated (Figure S11). Free **Probe 1** exhibited negligible fluorescence in the pH range from 1 to 12, suggesting that **Probe 1** was pH insensitive. As

expected, in the presence of 16 μM thiophenol, the fluorescence intensity increased with the pH range from 2.0 to 12.0, and the preferred pH range was 6.0-11.0. The weaker fluorescence response at pH ≤ 5.0 may attributed to the reduced nucleophilicity of thiophenol under these conditions. The obvious fluorescence decrease at pH > 11.0 was presumably due to the formation of disulfide,[12] thereby diminishing the concentration of thiophenol. The above studies demonstrated that **Probe 1** can detect thiophenol in environmental and biological studies.

3.6 Application potential of Probe **1**

Encouraged by the above-mentioned sensing performance of **Probe 1**, its practical applications including the detection of thiophenol in real-water samples, in serum, and on filter paper strips as well as in living cells were further demonstrated.

Detection of thiophenol in real-water samples. To verify the practicality of **Probe 1** in the environmental science, we applied **Probe 1** to quantify thiophenol concentrations in real water samples with the standard addition method.[37] The crude water samples form tap water and South Lake in Nanning city were passed through a microfiltration membrane (0.22 μm) before use. The pH value of the water samples were adjusted to pH 7.4 initially, and then the aliquots of the water samples were spiked with different concentrations of thiophenol (0, 1, 5 and 12 μM). The results were summarized in Table 2, and the resultant thiophenol recoveries were not less than 96%. These data led us to conclude that **Probe 1** has the potential for quantitative detection of thiophenol in real water samples.

Table 2. Determination of thiophenol concentration in water samples

Sample	Thiophenol spiked (μM)	Thiophenol recovered (μM)	Recovery (%)
South Lake water	0	Not detected	

	1	1.09±0.15	109
	5	5.32±0.10	106
	12	11.76±0.08	98
Tap water	0	Not detected	
	1	0.99±0.03	99
	5	5.40±0.18	108
	12	11.58±0.11	96

Detection of thiophenol by Probe **1** *coated filter paper strips.* Motivated by the strong fluorescence of **PB-NH$_2$** in solid state, the detection of thiophenol using **Probe 1** coated filter paper was carried out. Free **Probe 1** coated filter paper was essentially non-fluorescent. Upon addition of incremental concentrations of thiophenol, strong red fluorescence can be observed and the corresponding fluorescence enhancement at 621 nm can reach to 40 folds when 10^{-2} M thiophenol was added (Figure 6). Moreover, the fluorescence intensity at 621 nm was fairly linear in the thiphenol concentration range of 0.01-10 mM (Figure S12). To the best of our knowledge, this was the first paper strip that could be used for thiophenol detection, and this technology may provide a convenient method for large screening of environmental pollution samples.

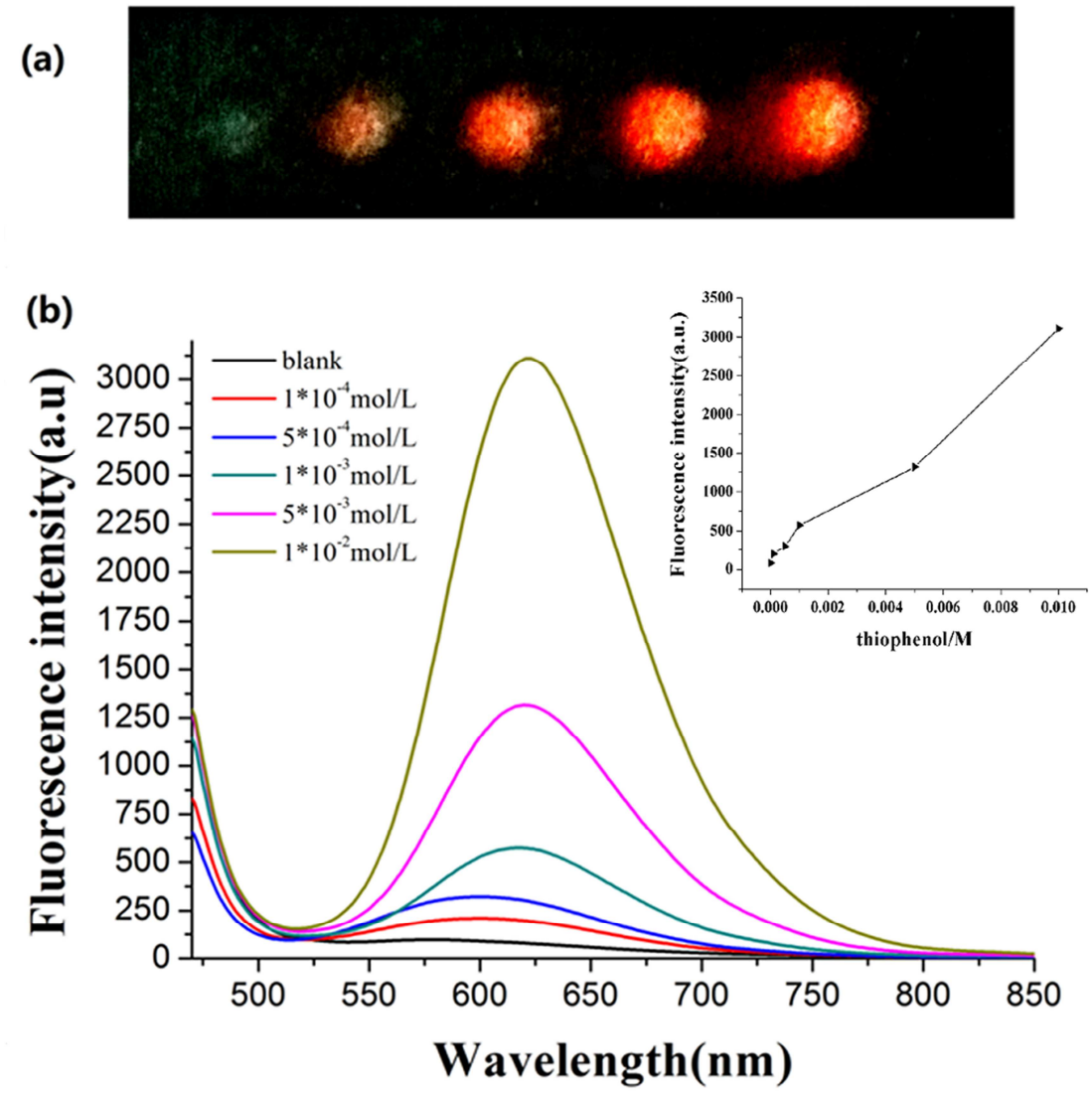


Figure 6. (a) Photograph of the corresponding samples on filter paper strip under UV 365 nm lamp; (b) Fluorescence spectra obtained in the titration of **Probe 1** with different equivalents of thiophenol on the filter paper strip.

Detection of thiophenol in Serum. To validate the biological applicability of **Probe 1**, the fluorescence titrations in the presence of fetal bovine serum (0.5 mL + 2.0 mL PBS buffer (10 mM, pH=7.4, containing 1 mM CTAB)) was carried out. Free **Probe 1** shows weak fluorescence in serum. Upon addition of thiophenol, strong red fluorescence peaked at 607 nm can be observed (Figure 7). The **Probe 1** shows a 225 fold enhancement of fluorescence intensity as a function of the concentration of added thiophenol and the corresponding

detection limit was calculate to be 5.9 nM (Figure S13). Thus, **Probe 1** is sensitive enough to detect thiophenol in biological fluids. It is worth noting that the fluorescence spectra induced by thiophenol in serum is slightly blue shifted (about 14 nm) when comparing the fluorescence spectra in PBS buffer (621 nm in PBS buffer vs. 607 nm in serum). This phenomenon might be explained by the accumulation of **Probe 1** into the hydrophobic cavities of the folding proteins in serum. Upon addition of thiophenol, the released dye **PB-NH$_2$** would stay in a relatively hydrophobic environment, as a result the non-radiative relaxation of **PB-NH$_2$** should be suppressed in some degree, thereby provoking an enhancement of the fluorescence intensity and a blue shift of the emission spectra. In addition, the above-mentioned speculation was further supported by the reaction kinetic study of **Probe 1** and thiophenol in serum (Figure S14-S15). The corresponding pseudo-first-order rate constant k_{obs}' was determined to be 0.0617 min^{-1}, which was much slower than the rate constant in PBS buffer (k_{obs} = 0.7727 min^{-1}), presumably due to the fact that the rigid barrel pocket of the protein in serum can preclude the effective collision between **Probe 1** and thiophenol.

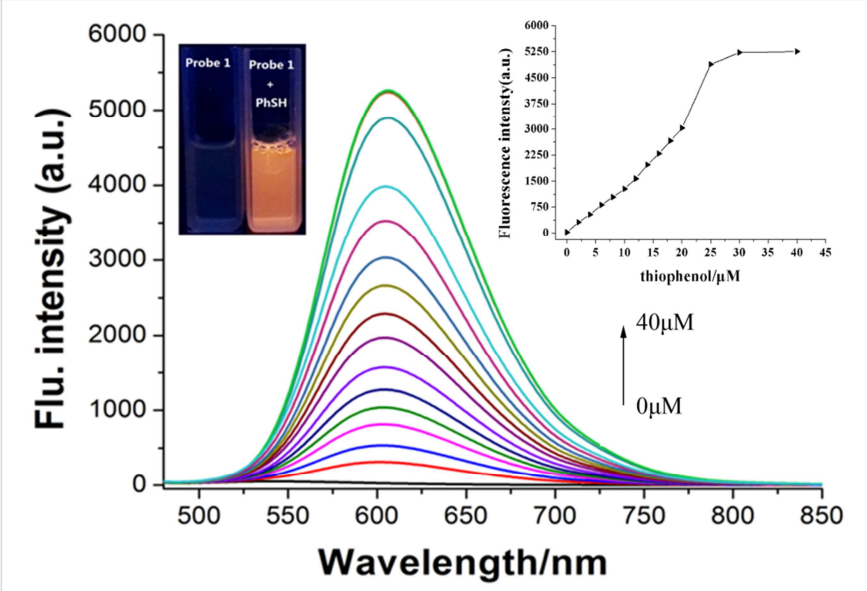


Figure 7. Fluorescence spectra of **Probe 1** upon addition of incremental amounts of thiophenol (0-40 μM) in the medium of fetal bovine serum in PBS buffer. Insert: photograph of **Probe 1** in

the absence and presence of thiophenol in the medium of serum in PBS buffer under the illumination by a UV 365 lamp.

Detection of thiophenol in living cells. Inspired by the excellent performance in aqueous solution, we sought out to investigate the capability of **Probe 1** to visualize thiophenol in living cells. Initially, we conducted MTT assays on HeLa cells to evaluate the cytotoxicity of **Probe 1**. The results suggested that this probe exhibited low cytotoxicity for living cells (Figure S16). When HeLa cells were pre-treated with 20 μM thiophenol for 20 min at 37°C then incubated with 10 μM **Probe 1** and 1 mM CTAB for additional 10 min, strong red fluorescence in the cell cytoplasm was observed (Figure 8c). In contrast, cells displayed non-noticeable fluorescence when cells only incubated with 10 μM **Probe 1** and 1 mM CTAB for 10 min (Figure 8a). This result indicated that **Probe 1** is cell-permeable and can be used for intracellular thiophenol detection.

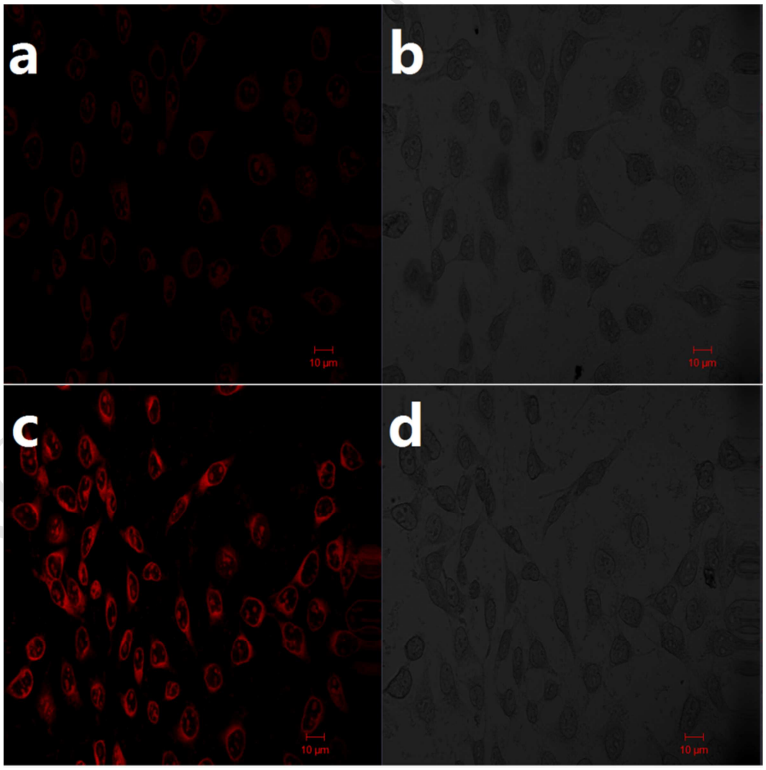


Figure 8. Confocal microscopic images of living HeLa cells. (a-b) HeLa cells incubated with 10 μM **Probe 1** and 1 mM CTAB for 15min; (c-d) HeLa cells pre-treated with 20 μM

thiophenol for 10 min then 10 μM **Probe 1** and 1 mM CTAB for additional 15 min. (a, c) fluorescence images; (b, d) Bright field images. Emission was collected at 600-650 nm window upon excited at 488 nm. Scale bar: 10 μm.

3. Experimental Section

2.1 Materials and Instruments

Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. Solvents were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. NMR spectra were recorded on a BRUKER 300 spectrometer with TMS as an internal standard. All accurate mass spectrometric experiments were performed on a Xevo G2 QTof MS (Waters, USA). UV-Vis absorption spectra were recorded on a TU-1901 (Puxi, P.R. China) spectrophotometer. Fluorescence spectra were recorded at room temperature using a HITACHI F-4600 fluorescence spectrophotometer with both the excitation and emission slit widths set at 5.0 nm. TLC analysis was performed on silica gel plates and column chromatography was conducted using silica gel (mesh 200-300), both of which were obtained from Qingdao Ocean Chemicals, China. HPLC analysis was performed on a Waters Acquity UPLC H-Class system (Milford, MA, USA) equipped with a quaternary solvent delivery system, a column oven, an auto sampler, and a photodiode array detector.

2.2 General Procedure for Spectral Measurements

A stock solution of **Probe 1** was prepared at 1 mM in DMF. Solutions of various testing species were prepared from Lys, Gln, Phe, His, Ala, Asp, Gly, Thr, Tyr, Met, Cys, Ser, Ile, Arg, Pro, Val, GSH, DTT, NaOAc, Na_2SO_4, $Na_2S_2O_3$, $NaHSO_3$, $NaNO_2$, $NaNO_3$, Na_3PO_4, Na_2S, $MgCl_2$, $ZnCl_2$, $CaCl_2$, KCl, H_2O_2, NaClO in twice-distilled water. Hcy was prepared in PBS buffer (10 mM, pH =7.4). 2-aminoethanethiol was prepared in ethanol. A typical test solution (10.0 mL) was prepared by placing 0.05 mL of **Probe 1** (1 mM), 5.0

mL of PBS buffer (20 mM, pH = 7.4, containing 2 mM Cetrimonium Bromide (CTAB)), and an appropriate aliquot of each analyte stock solution into an appropriate amount of twice-distilled water. The resulting solution was shaken well and kept at room temperature (25°C) for 20 min before recording its absorption and fluorescence spectra.

2.3 Determination of the fluorescence quantum yield

Fluorescence quantum yield of **PB-NH$_2$** was determined by using fluorescein (Φ_f = 0.79 in ethanol)[42] as a fluorescence standard. The quantum yield was calculated using the following formula (1):

$$\Phi_{F(x)}=\Phi_{F(s)}(A_S F_X/A_X F_S)(n_X/n_S)^2 \quad (1)$$

Where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts $_S$ and $_X$ refer to the standard and the unknown, respectively.

2.4 Prepare the test paper strips

Probe 1 (2 mg) was dissolved in 10 mL dichloromethane. Filter paper was immersed in the solution and then taken out to dry in air. Finally, the paper coated with **Probe 1** was cut into strips as the test paper for thiophenol detection.

2.5 Cell Culture and Fluorescence Imaging

HeLa cells were seeded in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin. The cells were incubated under an atmosphere of 5% CO_2 and 95% air at 37°C for 24 h. Cell imaging was performed with a Zeiss LSM 710 laser scanning confocal microscope. Before each experiment, cells were washed with PBS buffer 3 times. The cells were then incubated with **Probe 1** (10 μM), or pretreated with thiophenol (20 μM, 20 min) and further incubated with **Probe 1** (10 μM) at 37 °C (for 10 min). The samples were excited at 488 nm. Emission was collected at 600-650 nm.

2.6 Synthesis of compound **2**

In a 100 mL round-bottom flask, compound **1** (2.29 g, 10 mmol), n-bromobutane (13.6 g, 100 mmol), NaOH (0.8g, 20 mmol) and a catalytic amount of KI were dissolved in anhydrous DMSO (25 mL). The resulting mixture was stirred at 100 °C for 6 h under an argon atmosphere. After cooling to room temperature, the reaction mixture was poured into 300 mL water and extracted with dichloromethane (3×100 mL). The organic layer was separated and washed successively with brine and water, and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was further purified by silica gel flash chromatography using dichloromethane as an eluent to afford pure compound **2** (2.7g, 95% yield) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25-7.12 (m, 2H), 7.06 (d, J = 8.2 Hz, 1H), 6.92 (d, J = 8.1 Hz, 2H), 6.54 (s, 2H), 3.81 (d, J = 4.4 Hz, 5H), 1.89-1.79 (m, 2H), 1.56-1.46 (m, 2H), 0.99 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.8, 146.6, 145.0, 127.6, 127.3, 127.0, 125.5, 122.4, 116.0, 115.6, 106.7, 103.4, 55.5, 47.3, 29.0, 20.2, 13.8.

2.7 Synthesis of compound **3**

Dimethylformamide (0.17 mL, 2.4 mmol) was added to phosphorous oxychloride (0.2 mL, 2.4 mmol) at 0°C under argon atmosphere. The resulting mixture was allowed to stirring at this temperature for additional 15 min. Then a portion of compound **2** (570 mg, 2 mmol) (dissolved in 2 mL anhydrous DMF) was added to the cooled reagent with stirring. The mixture warmed to 60°C and stirred for 4 h, then poured into ice water (100 mL). The clear solution obtained was neutralized by $NaHCO_3$ solution (10%). The resulting sticky mass was extracted with dichloromethane (3×100 mL). The organic layers were separated, combined and washed successively with brine and water, dried over anhydrous Na_2SO_4 and vacuum evaporated. The residue was further purified by silica gel chromatography using petroleum ether/ethyl acetate (v/v, 4:1) as an eluent to give pure compound 3 (470 mg, 76% yield) as yellow powder. Mp: 81-82°C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.20 (s, 1H), 7.53 (s,

1H), 7.13 (dd, J = 16.8, 7.7 Hz, 2H), 7.01-6.85 (m, 2H), 6.37 (s, 1H), 3.90 (s, 5H), 1.84 (m, 2H), 1.48 (m, 2H), 0.97 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 187.2, 162.6, 152.4, 142.9, 127.4, 127.3, 126.7, 124.5, 123.5, 119.5, 116.0, 115.9, 98.6, 55.7, 47.8, 28.9, 20.1, 13.8. HRMS (ESI) m/z: calcd for $C_{18}H_{19}NO_2SNa$ $[M+Na]^+$, 336.1029; found 336.1028.

2.8 Synthesis of compound **4**

Aluminium powder (67.5 mg, 2.5 mmol) was added to anhydrous acetonitrile (5 mL) and stirred at room temperature for 5 min. To the slurry, iodine (394 mg, 1.55 mmol) was added in small portions and stirred under nitrogen atmosphere till the colour changed to yellow. Compound **3** (313 mg, 1 mmol) was dissolved in anhydrous acetonitrile (4 mL) and added to the slurry dropwise. The reaction mixture was then gently refluxed for 6 h, cooled to room temperature and poured into ice water (80 mL). The mixture was extracted with ethyl acetate (3×60 mL). Combined ethyl acetate extracts were washed with water, dried over anhydrous sodium sulphate. After removal of the solvent, the residue was further purified by silica gel chromatography using petroleum ether/dichloromethane (v/v, 4:1) as an eluent to afford compound 4 (162 mg, 54% yield) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.40 (s, 1H), 9.62 (s, 1H), 7.23-7.10 (m, 3H), 7.02-6.91 (m, 2H), 6.40 (s, 1H), 3.99-3.78 (m, 2H), 1.88-1.78 (m, 2H), 1.51-1.44 (m, 2H), 0.98 (t, J = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.3, 163.1, 153.2, 142.5, 130.9, 127.4, 124.0, 123.7, 116.2, 115.9, 114.4, 103.0, 48.0, 28.8, 20.0, 13.7. HRMS (ESI) m/z: calcd for $C_{17}H_{17}NO_2SNa$ $[M+Na]^+$, 322.0872; found 322.0873.

2.9 Synthesis of compound **5**

P-phenylenediamine (173 mg, 1.6 mmol) was dissolved in 12 mL absolute ethanol, and then a solution of compound **4** (100 mg, 0.33 mmol) in 5 mL ethanol was added dropwise. The resulting mixture was stirred at room temperature under an argon atmosphere and TLC was used to monitor the reaction. After completion, the precipitation was filtrated, washed by cool

ethanol and dried to afford compound **5** (95 mg, 74% yield) as red powder solids. Mp: 194-195°C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.78 (s, 1H), 7.56 (d, J = 8.8 Hz, 1H), 7.28 (d, J = 8.5 Hz, 1H), 7.14 (s, 1H), 7.22-7.14 (m, 2H), 7.07-6.96 (m, 2H), 6.79 (d, J = 8.8 Hz, 1H), 6.64 (d, J = 8.7 Hz, 1H), 5.53 (s, 1H), 3.93 (t, J = 6.9 Hz, 2H), 1.78–1.51 (m, 2H), 1.42 (dd, J = 14.9, 7.4 Hz, 2H),0.90 (t, J = 7.3 Hz,3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 159.8, 152.8, 149.8, 142.9, 132.6, 131.2, 128.3, 127.8, 124.3, 122.5, 117.1, 114.0, 109.5, 56.4, 47.5, 29.0, 19.6, 19.0, 14.0. HRMS (ESI) m/z: calcd for $C_{23}H_{23}N_3OSNa$ $[M+Na]^+$, 412.1460; found 412.1461.

2.10 Synthesis of **PB-NH$_2$**

In a 25 mL round-bottom flask, compound **5** (0.136g, 0.825 mmol) and DIEA (0.208 mL, 1.65 mmol) were dissolved in 4 mL 1,2-dichloroethane. The mixture was stirred at 80°C for 10 min, and then $BF_3 \cdot Et_2O$ (0.208 mL, 1.65 mmol) was added and the reaction mixture was left stirring at refluxing condition for 5h. After cooling to room temperature, the reaction mixture was diluted with 30 mL dichloromethane. The organic phase was washed successively with $NaHCO_3$ and brine water, dried over anhydrous sodium sulfate. After removal of the solvent, the residue was further purified by silica gel flash chromatography using petroleum ether/ethyl acetate (v/v, 1:5) as an eluent to give pure **PB-NH$_2$** (73 mg, 42% yield) as yellow powder. Mp: 98-100°C. ^{1}H NMR (300 MHz, CD_3CN) δ 8.33 (s, 1H),7.30-7.23 (m, 4H), 7.18 (dd, J = 7.6, 1.4 Hz, 1H),7.11-7.01 (m, 2H), 6.73 (d, J = 8.8 Hz, 1H), 6.58 (s, 1H), 4.46 (s, 1H) ,3.98 (t, J =7.1 Hz, 2H), 1.90 – 1.71 (m, 2H) ,1.47 (dd, J = 15.0, 7.4 Hz, 2H), 0.95 (t, J=7.4 Hz, 3H). ^{13}C NMR (75 MHz, CD_3CN) δ 160.1, 159.2, 154.3, 148.6, 142.2, 132.6, 129.0, 127.7, 127.1, 124.1, 123.8, 123.4, 115.1, 114.4, 103.8, 47.6, 28.3, 19.5, 12.9. HRMS (ESI) m/z: calcd for $C_{23}H_{23}BF_2N_3OS$ $[M+H]^+$, 438.1617; found 438.1620.

2.11 Synthesis of Probe **1**

In a 10 mL round-bottom flask, **PB-NH$_2$** (50 mg, 0.12 mmol), pyridine (0.018 mL,

0.23 mmol) were dissolved in 2 mL dichloromethane. The mixture was stirred at 0°C for 5 minute under an argon atmosphere, and then a solution of 2,4-dinitrobenzenesulfonyl chloride (61 mg, 0.23 mmol) in 2 mL dichloromethane was added. The resulting mixture was warmed to room temperature and stirred overnight. The reaction mixture was poured into water, and extracted with dichloromethane (2×30 mL). Organic layers were combined, dried over anhydrous sodium sulfate. After removal of the solvent, the residue was further purified by silica gel flash chromatography using petroleum ether/dichloromethane (v/v, 1:5) as an eluent to give pure compound **Probe 1** (71 mg, 93% yield) as red powder. Mp: 199-200°C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.38 (s, 1H), 8.90 (d, J = 2.2 Hz, 1H), 8.77 (s, 1H), 8.60 (dd, J = 8.7, 2.2 Hz, 1H), 8.28 (d, J = 8.7 Hz, 1H), 7.51 (d, J = 8.7 Hz, 2H), 7.43 (s, 1H), 7.26-7.22(m, 3H), 7.16 (dd, J = 18.6, 7.9 Hz, 2H), 7.03 (d, J = 7.5 Hz, 1H) ,6.63 (s, 1H), 3.98 (t, J = 7.0 Hz, 1H), 1.75–1.55 (m, 2H), 1.39 (dd, J = 14.9, 7.4 Hz, 2H), 0.87 (t, J = 7.4 Hz, 3H). 13C NMR (75 MHz, DMSO-d_6) δ 161.7, 159.9, 154.2, 150.0, 147.7, 141.3, 138.9, 136.0, 135.9, 131.5, 129.7, 127.8, 127.2, 127.0, 124.2, 123.8, 122.3, 121.1, 120.3, 117.0, 114.1, 110.9, 103.3, 47.0, 27.9, 19.1, 13.4. HRMS (ESI) m/z: calcd for $C_{29}H_{24}BF_2N_5O_7S_2Na$ $[M+Na]^+$, 692.1232; found 692.1237.

4. Conclusions

In summary, by introducing electron-rich phenothiazine moiety to the boranil dye, we have successfully developed a novel boron difluoride complex **PB-NH$_2$**. **PB-NH$_2$** displays much superior photophysical properties to those of classical BODIPYs, such as deep red emission, large Stokes shift and strong emission in solid state. By taking advantage of this robust platform, a novel fluorescent **probe 1** was designed and synthesized. Notably, **Probe 1** was ultrasensitive in response to thiophenol, the theoretical detection limit was calculated to be 1.4 nM and the experimental detection limit was 10 nM, to the best of our knowledge, it was the lowest experimental detection limit that has

ever been reported. More importantly, **Probe 1** was successfully applied to detect thiophenol in real-water samples, in serum, on filter paper strip and in living HeLa cells, suggesting that **Probe 1** has great potential for practical applications both in environmental and biological systems.

Acknowledgements

This work was supported by the Guangxi Natural Science Foundation (2016GXNSFBA380229) and "BAGUI Scholar" program of Guangxi Province of China.

Appendix A. Supplementary data

Electronic Supplementary Information (†ESI) available in the online version at http://dx.doi.org/xxx/xxx.

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- Novel amino phenothiazine boranil based fluorescent probe (**Probe 1**) for thiophenol was constructed.

- **Probe 1** features significant red fluorescence enhancement and remarkable Stokes shift in response to thiophenol in aqueous solution.

- **Probe 1** was ultrasensitive in response to thiophenol with a detection limit of 1.4 nM.

- **Probe 1** was successfully applied to detect thiophenol in real water samples, in serum, on filter paper strips and in living cells.