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AN INDOCYANINE-BASED TURN-ON FLUORESCENT PROBE FOR SPECIFIC DETECTION OF BIOTHIOLS

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Abstract – A novel heptamethine cyanine-based turn-on fluorescent probe Cy-DNBS was designed and synthesized, which was used successfully to detect biothiols with high selectivity and sensitivity. With the addition of biothiols, the probe displayed ~30-fold fluorescence enhancement at 625 nm. Meanwhile, the solution color changed remarkably from green to red, which provides a method to recognize biothiols by the 'naked eye'. The detection limit of Cys, GSH and Hcy are 2.24 nM, 1.99 nM and 4.46 nM, respectively.

INTRODUCTION

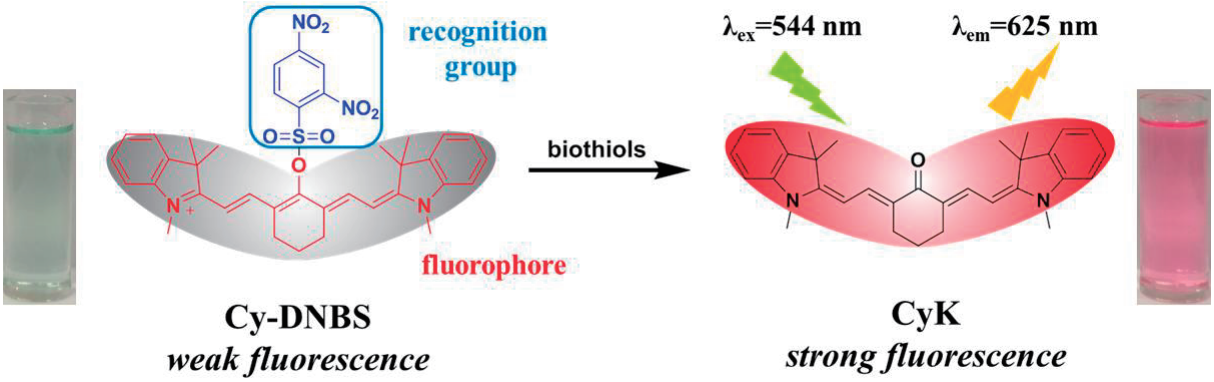
Biothiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play significant roles in maintaining the redox balance during many physiological and pathological processes.[1] In fact, a number of works have shown that abnormal level of thiols can result in some diseases,[2,3] such as the deficiency of Cys is related to liver damage, slow growth, skin lesions and hair depigmentation,[4,5] while high level of Hcy will increase the risk of Alzheimer's disease and cardiovascular.[6,7] GSH, the most abundant intracellular nonprotein thiol, is important in controlling oxidative stress, and the alteration of its level is connected with metabolism, stress responses, immune responses.[8,9] Therefore, developing an analytical method that can effectively detect biothiols is meaningful.

Over the past decades, many methods had been developed for biothiols' detection, such as electrochemical method, high performance liquid chromatography (HPLC) and mass spectrometry (MS).[10-12] Nonetheless, most of them require expensive instruments and complex operations. Compared with the above methods, fluorescence detection has been widely concerned by virtue of sensor simplicity, high sensitivity, good selectivity, noninvasiveness and convenient operation even in live cell and in vivo. In recent years, plenty of optical sensors have been developed to detect biothiols by several sensing strategies including Michael addition,[13,14] cleavage reactions,[8,15-18] cyclization reaction[19] and others.[20-25]

Despite all recent progess, it's still a challenge to develop novel simple probes, which can be synthesized easily and possess high sensitivity and selectivity.

Among well-known fluorophores, cyanine dyes are characteristic of high absorption coefficient, relatively long absorption and emission wavelength. Cyanine-ketone (CyK) derivatives are attractive dye because the polymethine π-conjugated system can be rationally modulated by introducing specific group on the meso-oxygen atom of the structure.[26] Several cyanine dyes functionalized at the meso-oxygen have been reported for detection of hydrazine[27] and H_2S.[28,29]

With these considerations in mind, a novel probe based on cyanine was designed and synthesized for the recognition of biothiols in PBS. The probe Cy-DNBS employed CyK as the fluorophore and 2,4-dinitrobenzenesulfonyl (DNBS) group as a recognition unit. The probe showed good sensitivity and selectivity to biothiols over other amino acids. Moreover, the solution color change provided a simple way for naked eye recognition of biothiols.



Scheme 1. The design and structure of probe Cy-DNBS

RESULTS AND DISCUSSION

The response time is an important parameter to evaluate a probe, therefore the time-dependent absorption and fluorescence intensity of probe Cy-DNBS towards thiols were investigated first. As shown in Figure 1A, in the prescence of 1 equiv. Cys, the absorption peak at 790 nm decreased significantly accompanied by the emergency of a new absorption peak at 544 nm, and the sensing process could reach to a plateau within 35 min. Meanwhile, an obvious isosbestic point at 610 nm was also observed, which distinctly demonstrated that a new species came into being upon the treatment of the probe with Cys. In addition, the fluorescence spectra displayed the same tendency with the absorption spectra upon addition of Cys. Probe Cy-DNBS is almost non-fluorescence originally. However, its fluorescence intensity increased significantly upon addition of 1 equiv. Cys and achieved the balance within 25 min (Figure 1B). Moreover, the reaction towards GSH and Hcy show similar results and changes of spectra can be

completely finished within 45 min (Figures S7, S8). Therefore, all of the following tests were performed after 45 min.

To investigate the sensitivity of probe Cy-DNBS to biothiols, fluorescence titration was carried out. As shown in Figure 2, the fluorescence intensity at 625 nm increased gradually with the increase of Cys concentration. And a good linear relationship between fluorescence intensity and the concentration of Cys was observed in the range of 0-4 μM with a correlation coefficient of 0.9896, displayed in the inset of Figure 2B. The detection limit for Cys was calculated to be 2.24 nM based on the signal to noise ratio, S/N=3. In addition, when Hcy and GSH were carried out the same experiments with the probe Cy-DNBS, similar phenomenon were observed (Figures S9, S10), and the detection limits for GSH and Hcy were evaluated to be 1.99 nM and 4.46 nM, respectively. Compared with the reported biothiols' probes, especially fluorescent probes having a DpNBS group,[1,7,15,16] our probe Cy-DNBS presented a relatively low detection limit, which indicating that probe Cy-DNBS possesses high sensitivity.

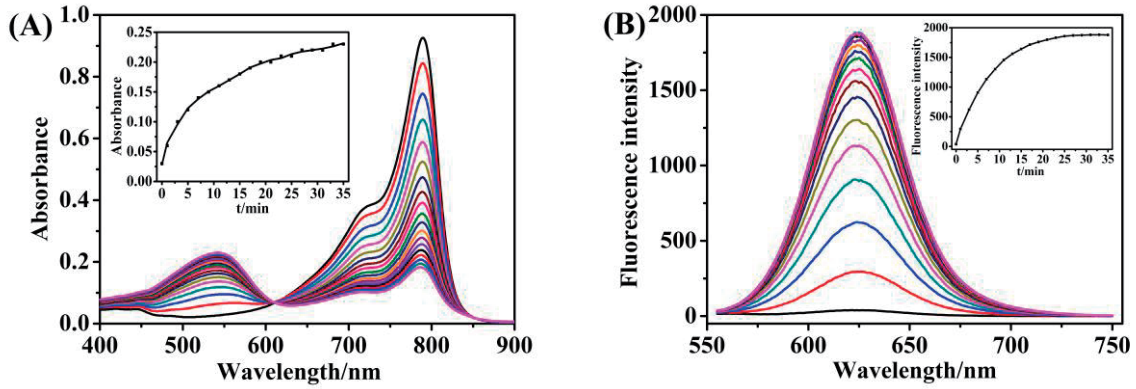


Figure 1. (A) Time-dependent absorption spectra of probe Cy-DNBS (10 μM) after adding 1 equiv. Cys. Inset: the absorbance at 544 nm along with time. (B) Time-dependent fluorescence spectra of probe Cy-DNBS (10 μM) after adding 1 equiv. Cys. Inset: the fluorescence intensity at 625 nm along with time.

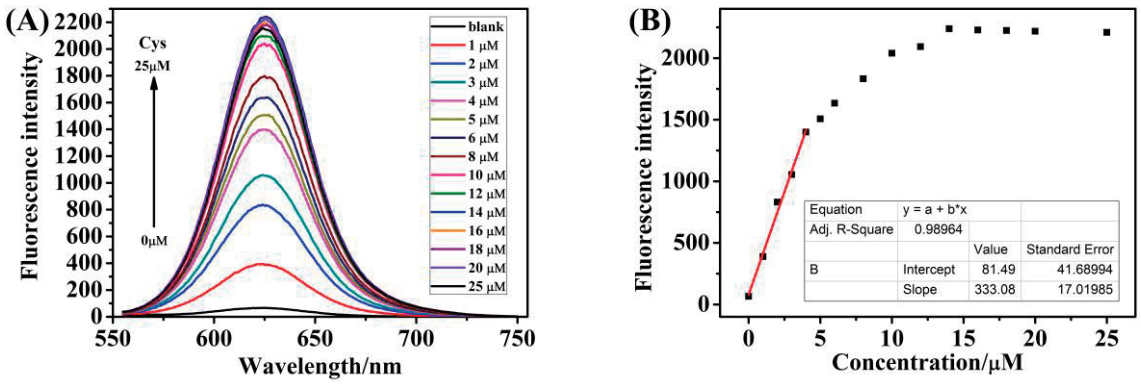


Figure 2. (A) Fluorescence spectra of probe Cy-DNBS (10 μM) treated with Cys, (B) Fluorescence intensity at 625 nm for probe Cy-DNBS (10 μM) as the function of the concentration of Cys.

It's significant that a probe has good selectivity towards specific analyte. Therefore the selectivity of probe Cy-DNBS for biothiols over other amino acids was investigated. As shown in Figure 3A, there was no significant change in absorption spectra with the addition of 10 equiv. other amino acids. However, the absorption peak at 790 nm decreased obviously and a new absorption peak at 544 nm arose dramatically with the addition of 1 equiv. Cys, Hcy, GSH. In the emission spectra, only biothiols induced a remarkable fluorescence enhancement with an excitation at 544 nm, while almost no change in fluorescence spectra was observed upon the treatment with the other amino acids over an hour (Figure 3B). Furthermore, the color of the solution changed distinctly from green to red, which can be identified by naked eye (Figure 3C). In addition, DNBS group has also been reported to be reacted with hydrogen sulfide (H_2S),[30] thus the reactivity of Cy-DNBS with H_2S was also examined. It can be seen from Figure 3, the absorption and fluorescence spectra only exhibited slight changes after addition of 10 equiv. HS^-, which can be neglected when compared with the changes caused by biothiols.

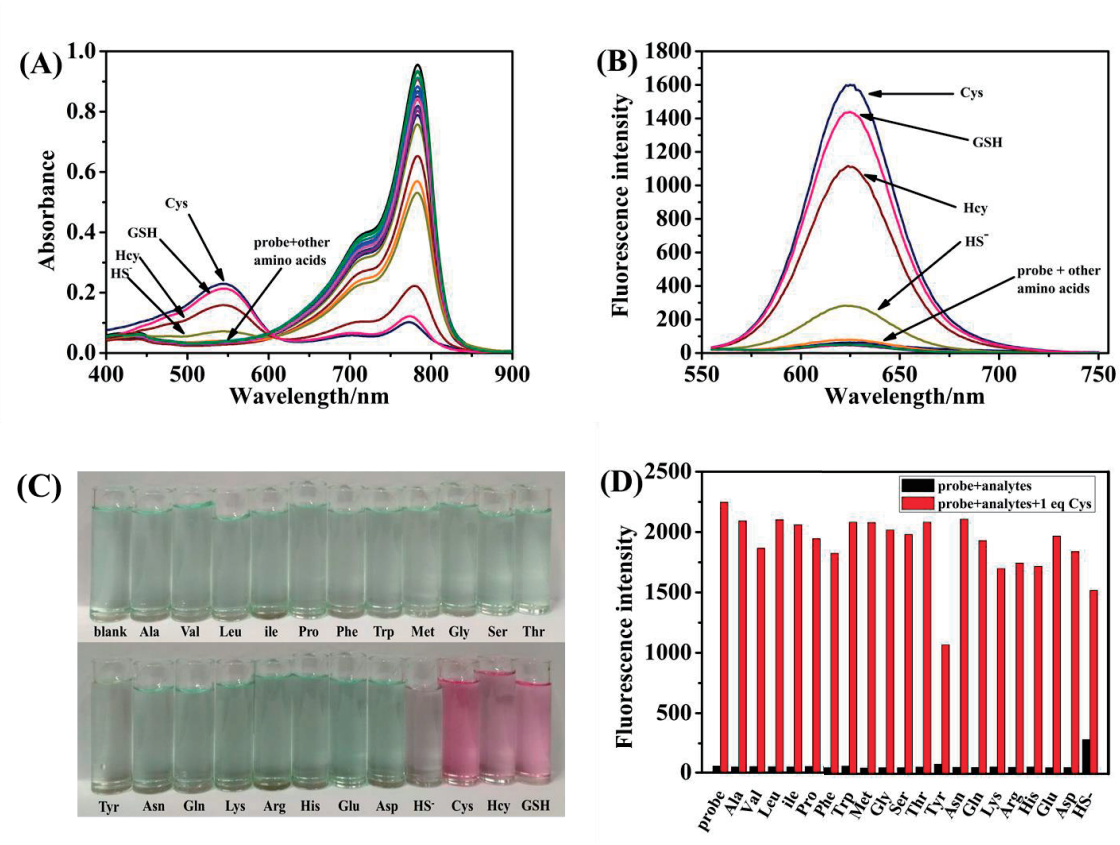


Figure 3. (A) Absorption spectra of probe (10 μM) in the presence of different analytes. (B) Fluorescence spectra of probe (10 μM) in the presence of different analytes. (C) Visual color changes of probe upon addition of various analytes. (D) Fluorescence intensity at 625 nm of probe in the presence of different analytes (black bars), and upon addition of 1 equiv. of Cys (red bars) (The other amino acids and HS^- were 10 equiv. except biothiols were 1 equiv).

In addition, to further explore the selectivity of the probe to biothiols in the presence of other nature amino acids and HS^-, interference experiments were conducted as follows: probe Cy-DNBS was first mixed with 10 equiv. other amino acids and HS^-, and 1 equiv. Cys was then added to the mixture. It can be seen from Figure 3D that upon the addition of 1 equiv. Cys, fluorescence intensity of the mixture was apparently enhanced, which demonstrated that probe Cy-DNBS possesses high selectivity toward biothiols even if it coexists with 10-fold concentration of other amino acids and HS^-.

For the sake of wide applications, the effect of pH on the fluorescence properties of probe Cy-DNBS was tested. First, the fluorescence intensity at 625 nm of probe Cy-DNBS at different pH was examined. The pH range from 2 to 12 was adjusted by 0.1 M hydrochloric acid solution and 0.1 M sodium hydroxide solution. It can be seen from Figure 4 that there was no significant change for the probe Cy-DNBS's fluorescence intensity with the change of pH value, which indicated that the probe was unchangeable in a wide pH range from 2 to 12. Subsequently, the response of the probe Cy-DNBS towards Cys at different pH was also investigated. After addition of 1 equiv. Cys, the fluorescence intensity was obviously enhanced in the pH range of 5-11, which illustrated that the probe can be used for the detection of biothiols in a wide pH range and has potential applications in physiological conditions. In the acidic conditions (pH 2-4), Cy-DNBS did not react with Cys because the detection of thiols is a process of nucleophilic substitution and the nucleophilic reaction is prevented under acidic conditions.[1]

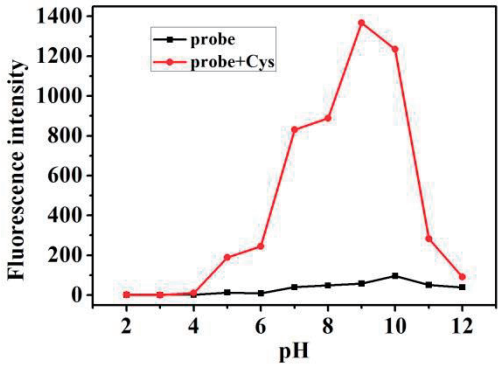
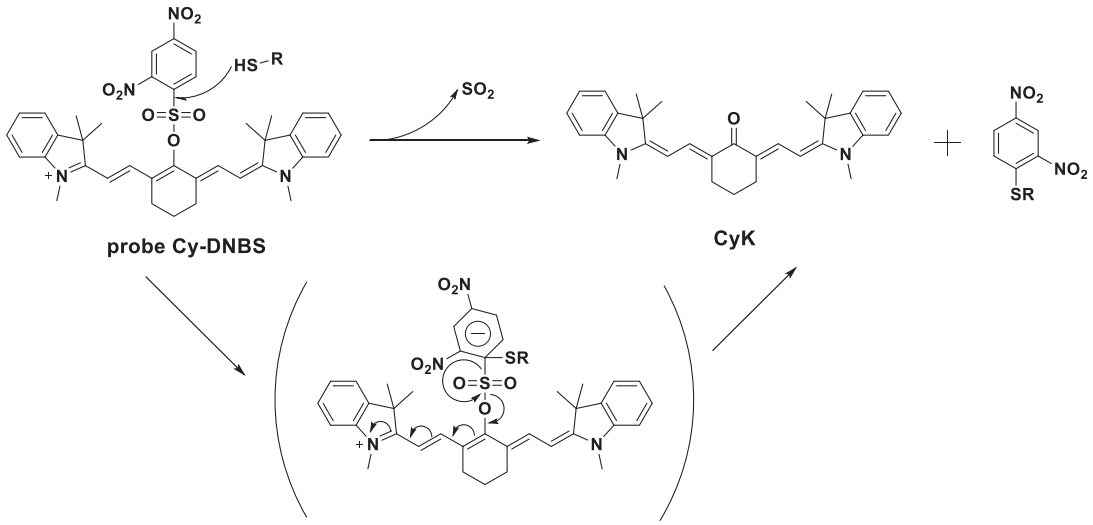


Figure 4. pH effect on the fluorescence properties of probe Cy-DNBS and the response of the probe towards Cys. The spectroscopic properties of probe Cy-DNBS was tested in ethanol-water (3:7, v/v).



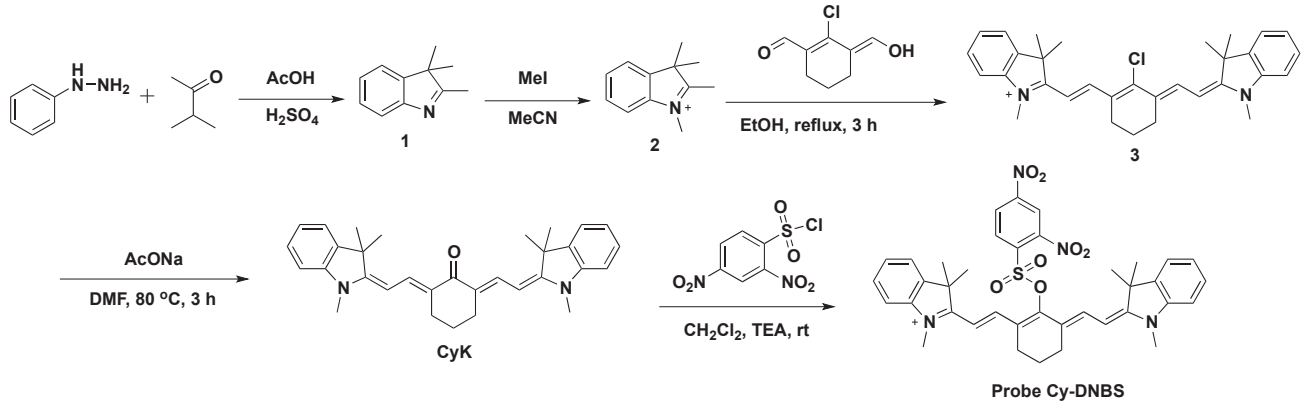
Scheme 2. Proposed responding mechanism of probe Cy-DNBS toward biothiols

In order to better understanding the interaction of the probe and biothiols, the response mechanism was studied. Several probes[1,15,16,31] with sulfonate ester linkage have been reported to selectively recognizing biothiols by a process of nucleophilic aromatic substitution. Therefore, we speculated that probe Cy-DNBS may also detect biothiols through the process of nucleophilic substitution. To confirm our assumption, the comparative experiments on absorption spectra and fluorescence spectra of CyK, probe Cy-DNBS and the reaction product of probe Cy-DNBS and biothiols were carried out. As shown in Figure S11, after treated with 1 equiv. thiol, probe Cy-DNBS exhibited an absorption peak shift from 790 nm to 544 nm, and a ~30-fold fluorescence response at 625 nm, which was in accordance with the absorption and fluorescence emission spectra of CyK. Furthermore, the results of mass spectrometry of probe Cy-DNBS treated with 1 equiv. Cys also indicated the generation of desired compound CyK (*m/z* 463.2725, for $[M-H]^-$) (Figure S12). These results suggest that the compound CyK was obtained indeed after treated with thiols. Thus, the response mechanism of probe Cy-DNBS toward biothiols was based on the nucleophilic substitution reactions as shown in Scheme 2.

In conclusion, a novel turn-on fluorescent probe for biothiols was successfully designed and synthesized based on heptamethine cyanine. The probe presents remarkable color changes upon the treatment with biothiols, which provides a simple way to detect thiols by naked eyes. Besides, the probe demonstrated good selectivity toward biothiols over other amino acids and the detection limit for Cys, GSH, Hcy was 2.24 nM, 1.99 nM and 4.46 nM, respectively. These results prove it to be an excellent probe for the selective detection of biothiols.

EXPERIMENTAL

The synthetic route of probe Cy-DNBS is illustrated in Scheme 3.



Scheme 3. The synthetic route of probe Cy-DNBS

Synthesis of CyK: This compound **1**, compound **2** and compound **3** were prepared according to our previously reported method.[32,33] A mixture of compound **3** (305 mg, 0.50 mmol) and sodium acetate (123 mg, 1.50 mmol) in anhydrous DMF (10 mL) was stirred at 90 °C for 3 h under nitrogen atmosphere. After cooling to room temperature, the solvent was removed and the crude product was purified by chromatography on silica gel (petroleum ether/EtOAc = 3:1, v/v) to afford compound CyK as a red solid (151 mg, 65% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.19 (d, *J* = 13.2 Hz, 2H), 7.19 (d, *J* = 7.6 Hz, 4H), 6.91 (t, *J* = 7.6 Hz, 2H), 6.70 (d, *J* = 8.0 Hz, 2H), 5.43 (d, *J* = 13.2 Hz, 2H), 3.21 (s, 6H), 2.62 (t, *J* = 5.6 Hz, 4H), 1.91-1.86 (m, 2H), 1.67 (s, 12H). ^{13}C NMR (100 MHz, CDCl3) δ (ppm): 186.42, 163.17, 144.57, 139.57, 132.73, 127.61, 126.80, 121.70, 120.45, 106.40, 92.51, 46.40, 29.26, 28.67, 25.82, 22.51. HRMS (ESI Positive) calc. for $C_{32}H_{36}N_2O$, $[M+H]^+$ 465.2900, found 465.2908. Fluorescence quantum yield: 10%.

Synthesis of probe Cy-DNBS: Compound CyK (464 mg, 1 mmol) was dissolved in 15 mL CH_2Cl_2 and stirred under the ice bath. Then triethylamine (101 mg, 1 mmol) was added to the solution and the mixture was stirred for 15 min. The solution of 2,4-dinitrobenzenesulfonyl chloride (798 mg, 3 mmol) in CH_2Cl_2 (5 mL) was added dropwise within 30 min under nitrogen protection. The reaction mixture kept stirring in ice bath for another 30 min. After that the mixture was warmed naturally to room temperature and stirred for 1 h. Then the mixture was poured into water (50 mL) and extracted with CH_2Cl_2 (25 mL × 2). The organic phase was dried over anhydrous sodium sulfate, filtered and concentrated to yield a black solid. The crude product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 20:1, v/v) to afford a green solid (353 mg, 28% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.38 (d, *J* = 8.5 Hz, 1H), 8.31 – 8.25 (m, 2H), 7.73 (d, *J* = 14.0 Hz, 2H), 7.37 (t, *J* = 7.7 Hz, 2H), 7.29 (d, *J* = 7.2 Hz, 2H),

7.22 (t, *J* = 7.4 Hz, 2H), 7.14 (d, *J* = 7.9 Hz, 2H), 6.16 (d, *J* = 14.0 Hz, 2H), 3.67 (s, 6H), 2.72 (m, 4H), 1.99 (m, 2H), 1.44 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 172.65, 156.46, 150.96, 148.83, 148.26, 147.42, 145.53, 142.66, 140.92, 140.12, 134.59, 134.26, 131.90, 128.81, 128.36, 125.59, 125.29, 123.88, 122.10, 120.47, 118.27, 110.93, 102.02, 53.51, 49.10, 31.90, 27.25, 25.67, 20.33. HRMS (ESI Positive) calc. for $C_{38}H_{39}N_4O_7S$, $[M]^+$ 695.2539, found 695.2529. Fluorescence quantum yield: 9%.

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