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A phenanthroimidazole-based fluorescent chemosensor for imaging hydrogen sulfide in living cells†

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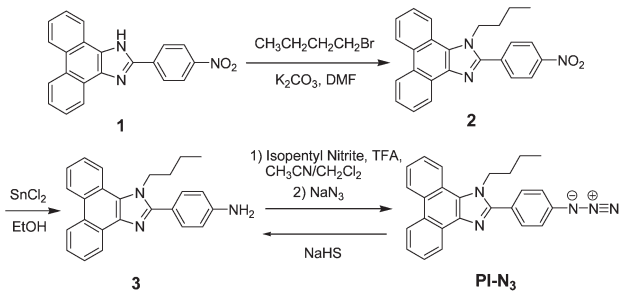
We have constructed a new fluorescence turn-on chemosensor for hydrogen sulfide based on a phenanthroimidazole scaffold, and the novel sensor is suitable for imaging hydrogen sulfide in living cells.

Introduction

Hydrogen sulfide (H_2S) has been recently recognized as the third gasotransmitter together with carbon monoxide (CO) and nitric oxide (NO).[1] Although long considered as a noxious gas with wide-ranging cytotoxic effects,[2] there is now an accumulation of scientific evidence that H_2S plays an important physiological role in many biological processes. In mammalian tissues, there are at least three known enzymes that produce H_2S:[3] cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MPST). These enzymes catalyze the conversion of cysteine or cysteine derivatives to H_2S within different organs and tissues. However, abnormal levels of H_2S are associated with many types of diseases including Alzheimer's disease,[4] liver cirrhosis,[5] Down's syndrome,[6] and diabetes.[7] Thus, it is of high interest to monitor H_2S levels in biosystems.

So far, several analytical techniques, including gas chromatograph,[8] colorimetric method,[9] electrochemical analysis,[10] and sulfide precipitation[11] have been developed for H_2S detection. Although these methods provide sensitive analysis, they require complicated sample preparation, sophisticated instrumentation, or destruction of tissues or cells. By contrast, fluorescence sensing *via* microscopy is noninvasive. Thus, it is suitable for studies of H_2S in living systems, and the development of fluorescent sensors for H_2S has attracted great attention.[12]

Herein, we report **PI-N$_3$** (Scheme 1) as a new fluorescent chemosensor for H_2S based on a phenanthroimidazole dye. We reasoned that the fluorescence of the phenanthroimidazole dye could be quenched by the azido group.[13] However, upon



Scheme 1 Design and synthesis of **PI-N$_3$**.

H_2S-mediated reduction of azides to amines,[12a–e,14] **PI-N$_3$** may be converted into amine **3**. This should elicit a significant change in fluorescence properties.

Results and discussion

The starting compound **1** was prepared according to a literature procedure.[15] Alkylation of compound **1** afforded compound **2**, which was further reduced to provide the key intermediate **3**. Finally, the desired product **PI-N$_3$** was synthesized from the intermediate **3** by the Sandmeyer reaction.[12a,16] All new compounds were characterized by the standard NMR spectroscopy, mass spectrometry, and elementary analysis.

With **PI-N$_3$** in hand, we examined its optical properties in the absence or presence of H_2S (NaHS was used as the hydrogen sulfide source in all experiments). The free chemosensor **PI-N$_3$** (5.0 μM) exhibited a maximal absorption band at around 358 nm (ε = 15 032 M^{-1} cm^{-1}) in pH 7.4, 25 mM PBS buffer–ethanol (7 : 3, v/v) at ambient temperature (Fig. S1†). As anticipated, the free **PI-N$_3$** showed relatively weak fluorescence (Φ = 0.030) due to the quenching effect of azido group. However, upon treatment with NaHS, a drastic enhancement in fluorescence intensity at around 423 nm was observed (Fig. 1).

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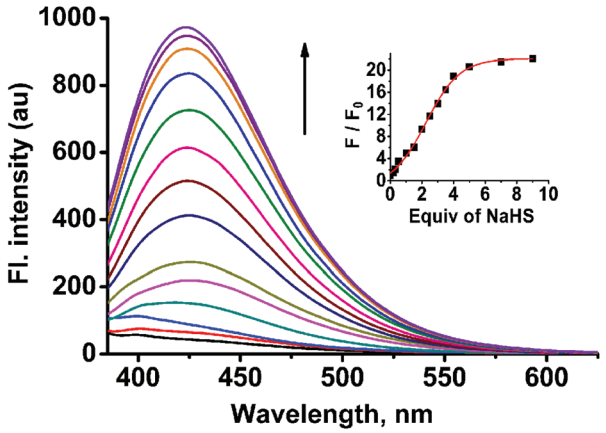

Fig. 1 Fluorescence spectra of **PI-N$_3$** (5.0 μM) in pH 7.4 PBS buffer–ethanol (7 : 3, v/v) in the absence or presence of NaHS (0–9 equiv.). Inset: fluorescence intensity ratio (F/F_0) changes at 423 nm of **PI-N$_3$** (5.0 μM) with the amount of NaHS. The spectra were recorded after incubation of the probe with NaHS for 3 min. Excitation at 350 nm.

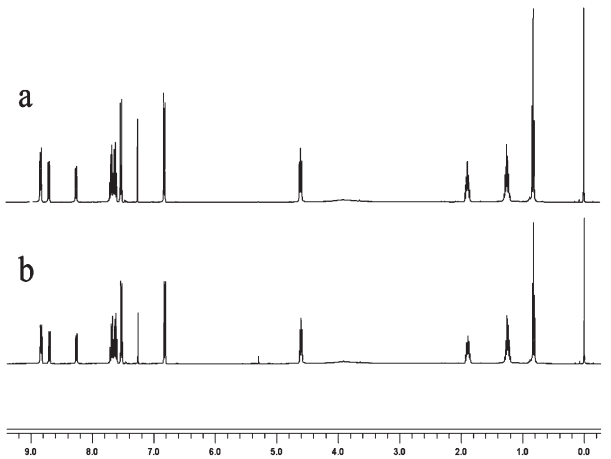

Fig. 2 ^{1}H NMR (400 MHz) spectra of the standard compound **3** (a) and the isolated product of **PI-N$_3$** + NaHS (b) in $CDCl_3$.

A maximal fluorescence enhancement (20-fold) was obtained when the concentration of NaHS reached 5.0 equiv. The detection limit for **PI-N$_3$** was calculated to be 8.79×10^{-7} M (Fig. S2†), indicating that the chemosensor is highly sensitive to H_2S.

To confirm the sensing process, we decided to study the product of **PI-N$_3$** + NaHS by both NMR spectroscopy and mass spectrometry. The product of **PI-N$_3$** + NaHS was isolated by a silica gel column and was then subjected to ^{1}H NMR analysis. The ^{1}H NMR of the resulting product is essentially identical to that of the authentic compound **3** (Fig. 2). In addition, the identity of the product of **PI-N$_3$** + NaHS was further characterized by the mass spectrometry analysis (Fig. S3†).

To better understand the photophysical properties of compound **3** and the chemosensor **PI-N$_3$**, they were examined by density function theory (DFT) calculations at the B3LYP/6-31G (d) level of the Gaussian 09 program. The molecular orbital plots of compound **3** and **PI-N$_3$** are shown in Fig. 3. For

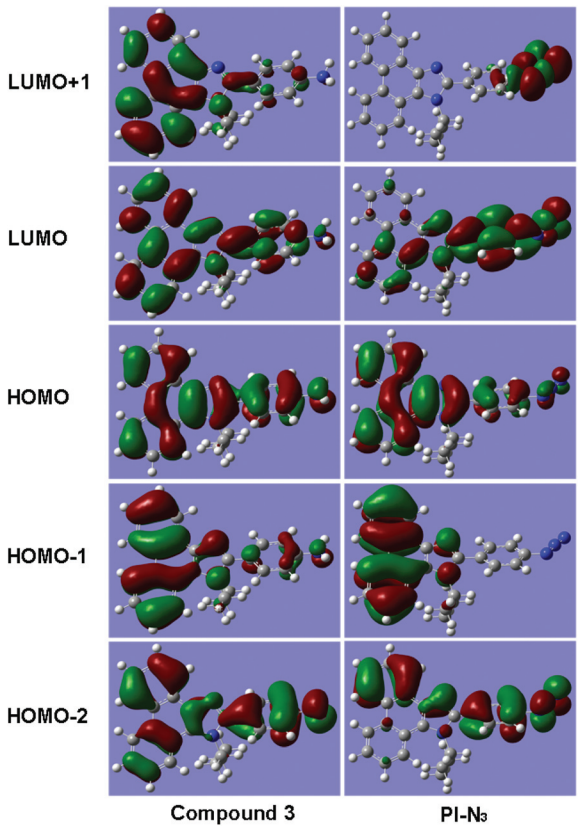

Fig. 3 Molecular orbital plots of **PI-N$_3$** and compound **3**.

compound **3**, the π electrons on both the HOMOs and LUMOs are essentially distributed in the entire phenanthroimidazole-benzene-amine backbone. By contrast, in the case of **PI-N$_3$**, the π electrons on HOMO and HOMO − 1 are primarily resided on the electron-donating phenanthroimidazole moiety, whereas those on LUMO, HOMO − 2 and LUMO + 1 are mainly located on the electron-withdrawing azido group. This indicates that **PI-N$_3$** bears efficient electron transfer from the phenanthroimidazole dye to the azido group, thus rendering the fluorescence relatively weak (Φ = 0.030). By contrast, the nearly complete overlap of electrons on the transition orbitals may induce the strong fluorescence emission for compound **3** (Φ = 0.62).[17]

The effect of pH on the fluorescence response of **PI-N$_3$** to H_2S was investigated. As shown in Fig. S4,† in the absence of NaHS, almost no change in fluorescence intensity was observed in the free chemosensor over a wide pH range of 5.0–8.5, indicating that the free chemosensor was stable in the wide pH range. Upon treatment with NaHS, the maximal fluorescence signal was observed in the pH range of 7–8.5, suggesting that the chemosensor functions properly at physiological pH.

The time courses of the fluorescence intensities of **PI-N$_3$** (5 μM) in the absence or presence of NaHS (1, 3, and 5 equiv.) in pH 7.4, 25 mM PBS buffer–ethanol (7 : 3, v/v) are displayed in Fig. 4. Notably, a drastic enhancement in fluorescence intensities was observed within a few seconds, and a plateau was reached in 3 minutes in the presence of 1, 3, or 5 equiv. of

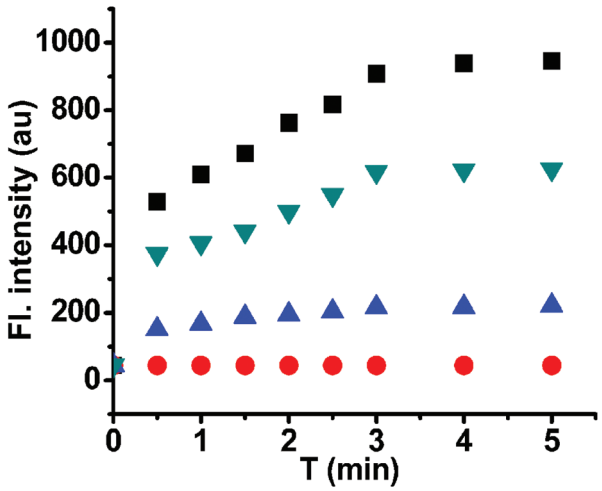

Fig. 4 Reaction-time profiles of **PI-N$_3$** (5 μM) in the absence [●] or presence of NaHS (5 μM [▲], 15 μM [▼], 25 μM [■]). The fluorescence intensities at 423 nm were continuously monitored at time intervals in pH 7.4, 25 mM PBS buffer–ethanol (7 : 3, v/v). Time points represent 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 min.

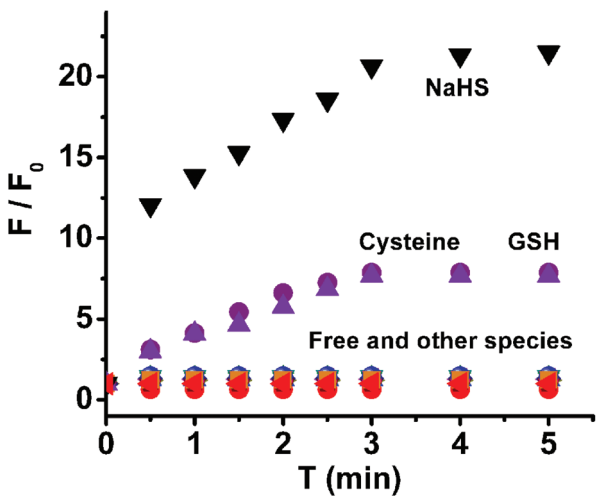

Fig. 5 Reaction-time profiles for chemosensor **PI-N$_3$** (5 μM) to various relevant species (150 μM for H_2O_2, ClO^-, $S_2O_3^{2-}$, 1 mM for citrate, Cl^-, CO_3^{2-}, F^-, HCO_3^-, HPO_4^{2-}, N_3^-, NO_2^-, OAc^-, SO_4^{2-}, cysteine, GSH, and 25 μM for NaHS) in pH 7.4, 25 mM PBS buffer–ethanol (7 : 3, v/v).

NaHS. We further examined the time courses of the fluorescence intensities of the chemosensor in the presence of 5 equiv. NaHS in different solvent systems. As shown in Fig. S5,† in DMF, the fluorescence response of the chemosensor was very fast, and the maximal fluorescence signal was reached within 4 min. The same behavior was observed using EtOH as the solvent (Fig. S6†). Thus, these studies suggest that the rapid fluorescence response of the chemosensor may render it suitable for real time detection of H_2S, which fluctuates fast in living systems.

PI-N$_3$ was treated with various relevant analytes including anions, reactive oxygen species, reducing agents, small-molecule thiols, and NaHS in pH 7.4, 25 mM PBS buffer–ethanol

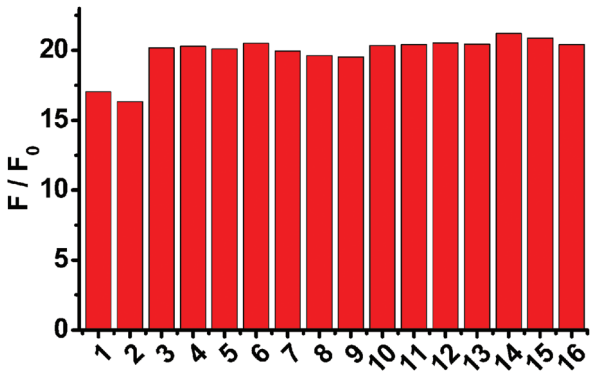

Fig. 6 The fluorescent responses of the chemosensor **PI-N$_3$** (5 μM) to NaHS in the presence of various relevant species (150 μM for H_2O_2, ClO^-, $S_2O_3^{2-}$, 1 mM for citrate, Cl^-, CO_3^{2-}, F^-, HCO_3^-, HPO_4^{2-}, N_3^-, NO_2^-, OAc^-, SO_4^{2-}, cysteine, GSH, and 25 μM for NaHS) in pH 7.4, 25 mM PBS buffer–ethanol (7 : 3, v/v). 1. NaHS + ClO^-; 2. NaHS + H_2O_2; 3. NaHS + citrate; 4. NaHS + Cl^-; 5. NaHS + CO_3^{2-}; 6. NaHS + F^-; 7. NaHS + HCO_3^-; 8. NaHS + HPO_4^{2-}; 9. NaHS + N_3^-; 10. NaHS + NO_2^-; 11. NaHS + OAc^-; 12. NaHS + $S_2O_3^{2-}$; 13. NaHS + SO_4^{2-}; 14. NaHS + cysteine; 15. NaHS + GSH; 16. NaHS. The spectra were recorded after incubation of the probe with various relevant species for 3 min.

(7 : 3, v/v) to investigate the selectivity over a period of 5 min. As exhibited in Fig. 5, introduction of representative species including ClO^-, H_2O_2, citrate, Cl^-, CO_3^{2-}, F^-, HCO_3^-, HPO_4^{2-}, N_3^-, NO_2^-, OAc^-, $S_2O_3^{2-}$, and SO_4^{2-} elicited essentially no fluorescence response over 5 min. Although the response of the probe to cellular abound thiols such as GSH and cysteine is time-dependent, a maximal response (<8.0 fold) was obtained in 3 min. By contrast, importantly, introduction of NaHS caused a large fluorescent enhancement (20-fold) in 3 min. These data indicate that the chemosensor is selective for H_2S over other tested species including GSH and cysteine. In addition, we further examined the fluorescence response of the probe toward NaHS in the presence of other potentially competing species. The other species only displayed minimum interference (Fig. 6). This suggests that **PI-N$_3$** is potentially useful for sensing NaHS in the presence of other related species.

We then proceeded to examine the capability of the chemosensor for monitoring changes of H_2S levels in live cells. HeLa cells were incubated with 5 μM **PI-N$_3$** for 20 min, and then treated with various concentrations (0, 20, or 50 μM) of NaHS. As shown in Fig. 7b, the cells incubated with only the chemosensor displayed relatively weak fluorescence. However, the cells load with both the chemosensor and NaHS exhibited significant fluorescence (Fig. 7d and f). The comparison among Fig. 7b, 7d, and 7f reveals a dose-dependent fluorescence enhancement. Thus, these results establish that **PI-N$_3$** is cell membrane permeable and capable of sensing H_2S in living cells.

Conclusions

In summary, we have designed and synthesized a new fluorescent chemosensor, **PI-N$_3$**, for H_2S based on a

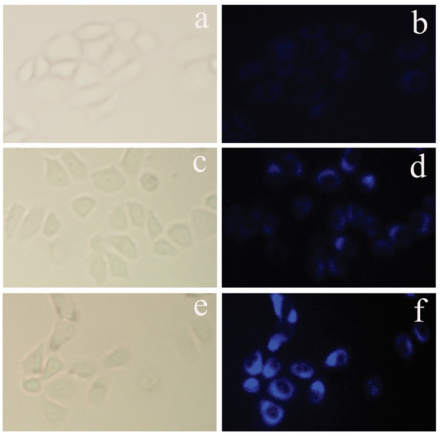

Fig. 7 (a) Bright-field image of live HeLa cells incubated with only **PI-N$_3$** (5 μM) for 20 min; (b) fluorescence image of (a); (c) bright-field image of live HeLa cells incubated with **PI-N$_3$** (5 μM) for 20 min, then with NaHS (4 equiv.) for 15 min; (d) fluorescence image of (c); (e) bright-field image of live HeLa cells incubated with **PI-N$_3$** (5 μM) for 20 min, then with NaHS (10 equiv.) for 15 min; (f) fluorescence image of (e).

phenanthroimidazole scaffold. Notably, **PI-N$_3$** is highly reactive to H_2S with a very rapid response. The other attributes of the novel fluorescent chemosensor include high sensitivity, high selectivity, and function well at physiological pH. Significantly, we have further demonstrated that the chemosensor is suitable for monitoring variations of H_2S levels in living cells.

Experiment section

Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, and all melting points were uncorrected. Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer. NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard. Elemental analyses were performed in an Euro EA 3000 elemental analyzer. Electronic absorption spectra were obtained on a Labtech UV Power PC spectrometer. Photoluminescent spectra were recorded at room temperature with a HITACHI F4600 fluorescence spectrophotometer. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Preparation of the test solution

The stock solution of **PI-N$_3$** was prepared at 0.5 mM in ethanol. The solutions of various testing species were prepared from NaCl, NaF, NaN_3, $Na_2S_2O_3$, $NaNO_2$, CH_3COONa, Na_2CO_3, $NaHCO_3$, Na_2HPO_4, Na_2SO_4, GSH, cysteine, H_2O_2, NaClO, sodium citrate, and NaHS in the twice-distilled water. The test solution of **PI-N$_3$** (5.0 μM) in 3 mL buffered aqueous solution (containing 30% ethanol as a co-solvent, 25 mM PBS buffer, pH 7.4) was prepared by placing 0.03 mL of the **PI-N$_3$** stock solution and 0.87 mL ethanol in 2.1 mL of buffered aqueous solution (containing 30% ethanol as a co-solvent, 25 mM PBS buffer, pH 7.4). The resulting solution was shaken well at room temperature before recording the spectra. Unless otherwise noted, for all measurements, the excitation wavelength was 350 nm, the excitation slit widths were 5 nm, and emission slit widths 5 nm.

Cell culture and fluorescence imaging

HeLa cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were plated on 12-well plates and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with PBS buffer. The HeLa cells were then incubated with **PI-N$_3$** (5 μM) in the culture medium for 20 min at 37 °C. After washing with PBS three times to remove the remaining chemosensor, the cells were further incubated with NaHS (20 μM or 50 μM) for 15 min at 37 °C and imaged with a Nikon Eclipse TE300 equipped with a CCD camera.

Synthesis of 1-butyl-2-(4-nitrophenyl)-1*H*-phenanthro[9,10-*d*]-imidazole (2). Compound **1** (339 mg, 1.00 mmol), 1-bromobutane (476 mg, 3.50 mmol), and K_2CO_3 (276 mg, 2.00 mmol) were dissolved in anhydrous DMF (6 mL), and the reaction mixture was heated at 50 °C for 6 hours under N_2 atmosphere. The reaction mixture was then poured into 40 mL H_2O, and then extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. Purification by silica gel column chromatography (CH_2Cl_2 : petroleum = 1 : 5 to 2 : 1) gave 324 mg (0.820 mmol, 82%) of compound **2** as a yellow solid: m.p. = 202–204 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (d, J = 8 Hz, 1H, ArH), 8.77 (d, J = 7.2 Hz, 1H, ArH), 8.71 (d, J = 8 Hz, 1H, ArH), 8.43 (d, J = 9.2 Hz, 2H, ArH), 8.27 (d, J = 7.6 Hz, 1H, ArH), 7.98 (d, J = 8.8 Hz, 2H, ArH), 7.74–7.65 (m, 4H, ArH), 4.66 (t, J = 7.2 Hz, 2H, NCH_2), 1.97–1.89 (m, 2H, NCH_2CH_2), 1.28–1.18 (m, 2H, CH_2CH_3), 0.82 (t, J = 7.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.0, 148.2, 130.9, 129.6, 128.3, 127.5, 127.0, 126.0, 125.4, 124.6, 124.0, 123.1, 122.6, 120.9, 47.1, 32.3, 19.6, 13.5. MS(ESI) m/z = 396.1 $[M + H]^+$. HRMS(EI) calcd for $C_{25}H_{21}O_2N_3$, 395.1628; Found 395.1654. Anal. calcd for $C_{25}H_{21}O_2N_3$: C, 75.93; H, 5.35; N, 10.63. found: C, 76.26; H, 5.51; N, 10.93.

Synthesis of 4-(1-butyl-1*H*-phenanthro[9,10-*d*]imidazol-2-yl)-aniline (3). Compound **2** (160 mg, 0.410 mmol) was dissolved in 30 ml of ethanol, and then $SnCl_2{\cdot}2H_2O$ (632 mg, 2.80 mmol) was added. The reaction mixture was refluxed for 8 h and then cooled to room temperature. Subsequently, the reaction mixture was adjusted to basic pH with saturated $NaHCO_3$, and then extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. Purification by silica gel column chromatography

(CH_2Cl_2 : ethanol 100 : 1 to 50 : 1) afforded 90 mg (0.240 mmol, 61%) of compound **2** as a white solid: m.p. = 162–164 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (d, J = 8.4 Hz, 2H, ArH), 8.70 (d, J = 8 Hz, 1H, ArH), 8.26 (d, J = 8.4 Hz, 1H, ArH), 7.71–7.60 (m, 4H, ArH), 7.53 (d, J = 8.8 Hz, 2H, ArH), 6.82 (d, J = 8.8 Hz, 2H, ArH), 4.61 (t, J = 7.6 Hz, 2H, NCH_2), 1.93–1.85 (m, 2H, NCH_2CH_2), 1.29–1.20 (m, 2H, CH_2CH_3), 0.82 (t, J = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.2, 147.7, 131.3, 128.9, 128.0, 127.2, 126.7, 125.8, 125.4, 124.6, 124.4, 123.4, 123.0, 122.7, 120.6, 114.9, 46.8, 32.3, 19.6, 13.6. MS(ESI) m/z = 366.1 $[M + H]^+$. HRMS(EI) calcd for $C_{25}H_{23}N_3$, 365.1886; Found 365.1880. Anal. calcd for $C_{25}H_{23}N_3$: C, 82.16; H, 6.34; N, 11.50. found: C, 82.10; H, 6.45; N, 11.20.

Synthesis of 2-(4-azidophenyl)-1-butyl-1*H*-phenanthro[9,10-*d*]-imidazole (PI-N_3). Compound **3** (55 mg, 0.15 mmol) was dissolved in 15 mL CH_3CN : CH_2Cl_2 (2 : 1), and then trifluoroacetic acid (24 mg, 0.21 mmol) was added to give a red precipitate. Subsequent addition of iso-amyl nitrite (21 mg, 0.18 mmol) gave a yellow solution. After stirring for 2 h at 0 °C temperature, sodium azide (21 mg, 0.32 mmol) was added and the solution was allowed to stir for an additional 1 h. The reaction mixture was then poured into 40 mL saturated aqueous $NaHCO_3$. The layers were separated and the aqueous layer was washed with an additional 2 × 30 mL CH_2Cl_2. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by silica column chromatography (CH_2Cl_2 : petroleum 1 : 5 to 1 : 1) to yield compound **PI-N_3** (30 mg, 51%): m.p. = 132–134 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (d, J = 8.4 Hz, 1H, ArH), 8.78 (d, J = 8 Hz, 1H, ArH), 8.70 (d, J = 8.0 Hz, 1H, ArH), 8.25 (d, J = 8.0 Hz, 1H, ArH), 7.74 (d, J = 8.4 Hz, 2H, ArH), 7.69 (d, J = 6.8 Hz, 2H, ArH), 7.66 (d, J = 4.8 Hz, 1H, ArH), 7.62 (d, J = 7.2 Hz, 1H, ArH), 7.21 (d, J = 8.4 Hz, 2H, ArH), 4.60 (t, J = 7.6 Hz, 2H, NCH_2), 1.93–1.86 (m, 2H, NCH_2CH_2), 1.28–1.18 (m, 2H, CH_2CH_3), 0.82 (t, J = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.8, 141.3, 131.6, 129.2, 128.1, 127.3, 126.8, 126.2, 125.6, 124.8, 124.5, 123.4, 123.0, 122.6, 120.7, 119.3, 46.8, 32.4, 19.6, 13.5. MS(ESI) m/z = 392.0 $[M + H]^+$. HRMS(EI) calcd for $C_{25}H_{21}N_5$, 391.1791; Found 391.1784. Anal. calcd for $C_{25}H_{21}N_5$: C, 76.70; H, 5.41; N, 17.89. found: C, 76.78; H, 5.40; N, 17.69.

Acknowledgements

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