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PAPER

Highly selective, naked-eye and fluorescent "off-on" probe for detection of histidine/histidine-rich proteins and its application in living cell imaging†

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A highly selective colorimetric and fluorescence enhanced probe **S1** (M2@Cu) for histidine and histidine-rich proteins has been developed. In neutral aqueous ethanol solution, probe **S1** can selectively detect histidine out of twenty DNA encoded amino acids by showing a color change from brownish red to light green, and with a fluorescence enhancement up to 99-fold at 537 nm, simultaneously.

Introduction

Despite the fact that significant progress has been made in the development of sensitive and selective fluorescent sensors and probes for molecular imaging, the successful examples are mainly limited to the detection of metal ions.[1] The selective probes for imaging of biologically important small organic molecules and macromolecules are highly valuable for biomedical research. Nevertheless, the development of such probes is formidably challenging and examples are limited.[2,3]

One of 20 kinds of natural amino acids, histidine (His), plays crucial roles in a number of proteins through key metal–ligand coordination or acid–base catalysis with the imidazole moiety, including carbonic anhydrases,[4] MMP-2,[5] histidine kinases[6] and so on. Furthermore, importantly, histidine is essential for children's growth and the repair of human tissues.[7] Its overexpression has been associated with a number of diseases, including AIDS,[8] chronic kidney disease,[9] Alzheimer's disease[10] and cancer[11] Therefore, the detection of histidine has received considerable attention in recent years. Several methods have been developed for the determination of histidine, including voltammetry,[12] liquid chromatography[13] and colorimetry.[14] While voltammetry and liquid chromatography based methods display high selectivity, they are not feasible for measuring spatial distribution and usually require significant laborious work compared to methods utilizing optical tactics. Fluorescent probes are more favorable in histidine detection as they show results of high selectivity and sensitivity.[15] In recent years, a series of fluorescent probes for histidine detection has been reported.[16] For example, Fabbrizzi[16d] and co-workers developed noncovalent fluorescent probes for histidine detection using Cu(II) macrocyclic complexes. Kwok Yin Wong[16c] and co-workers also employed a photoluminescent iridium(III) complex to detect histidine. Nevertheless, a more convenient, and practical imaging method is still in demand, particularly for real samples such as histidine-related proteins and living cells. Herein, we wish to report a readily available Cu^{2+}- naphthalimide based ligand complex (**S1**) as a fluorescent and colorimetric probe for specific recognition of histidine, histidine-rich proteins and living cell imaging. The ligand (**M2**) was first developed by our group[17] as a selective and sensitive ICT fluorescent chemosensor for Cu^{2+} ($K_a = 6.3 \times 10^5$). It chelates Cu^{2+} ions *via* a 1 : 1 stoichiometry in ethanol-water (60 : 40, v/v, 50 mM HEPES, pH 7.2) solution with the maximum absorption of 457 nm, and the maximum emission moved from 523 nm to 475 nm after Cu(II) binding to the N atom in naphthalene due to the ICT blue shift (Fig. S1†). Upon chelation, the color of the ligand solution changed from green to brownish red, while the fluorescence was switched off. We envision that the resulting Cu^{2+}–ligand complex (**S1**) could be used for histidine recognition.

Result and discussion

On the basis of the above rationale, probe **S1** was developed. Firstly, 4-bromo-5-nitro-1,8-naphthalimide **4** was chosen as starting material. Compound **3** was synthesized by the treatment of 4- bromo-5-nitro-1,8-naphthalimide **4** with *n*-butylamine in ethanol in 80% yield after refluxing for 2 h and recrystallization, as shown in Scheme 1. **M2** was synthesized from compound **3** with N-Boc-ethylenediamine and 2-chloromethylpyridine orderly in total 40% yield after column chromatography. Afterwards, **M2** was treated with cupric perchlorate ($Cu(ClO_4)_2$) to afford the fluorescent probe **S1**.

The influence of pH on the fluorescence of **S1** was first determined by fluorescence titration in an ethanol–water (60 : 40, v/v) solution. As shown in Fig. 1a, the fluorescence of **S1** at 537 nm showed a little increase from pH 2.7 to pH 3.9, then rapidly

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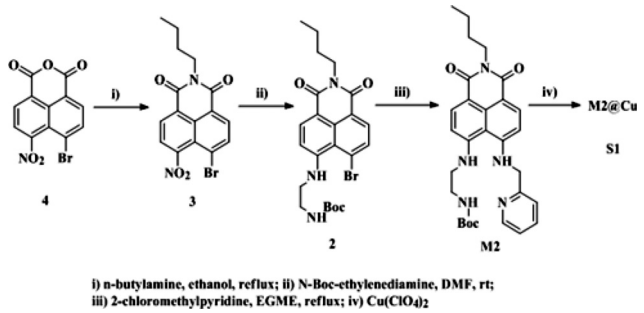
†Electronic supplementary information (ESI) available: Spectra of compounds **3**, **2**, **M2** and **S1**. See DOI: 10.1039/c2ob06520g



Scheme 1 Synthesis of fluorescent probe **S1**.

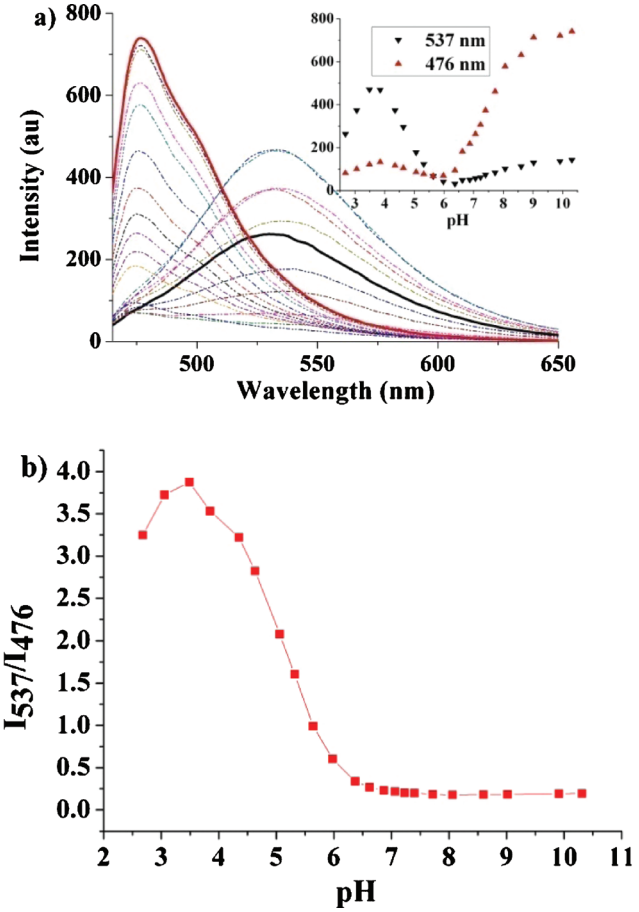


Fig. 1 (a) Fluorescent emission spectra of **S1** in the ethanol–water (60 : 40, v/v) solution from pH 2.56 to 11.15. Excitation wavelength = 457 nm. Inset: Influence of pH on the fluorescence of **S1** at 537 nm (black) and 476 nm (red). (b) Influence of pH on the fluorescence of **S1** in the ethanol–water (60 : 40, v/v) solution.

decreased by 12-fold when the pH changed from 3.9 to 6.4. The fluorescence intensity remained unaffected beyond pH 6.5. As we know, with increasing pH from 3.9 to 6.4, the protonated pyridine would lose the proton that induced the pyridine binding to Cu^{2+} leading to the emission decrease at 537 nm, which is due to the ligand-to-metal charge transfer (LMCT) because of the d^9 system of the Cu^{2+} ion[18] Moreover, the fluorescence of **S1** at 476 nm remained unaffected between pH 2.7 to pH 6.4, and then increased dramatically from pH 6.4 to pH 10.3, which was induced by an internal charge transfer (ICT) mechanism caused

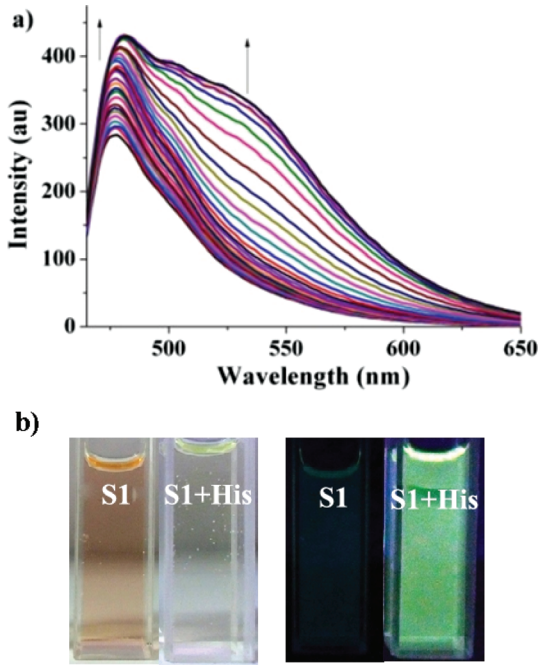


Fig. 2 (a) Relative fluorescent emission spectra of **S1** (10 μM) with histidine in ethanol–water solution (60 : 40, v/v, 50 mM HEPES buffer, pH 7.2) with 457 nm excitation. (b) Imaging of the color and fluorescent change of probe **S1** before and after adding His.

by Cu(II) binding.[17] Additionally, Fig. 1b shows the influence of pH on the fluorescence intensity of **S1** with the ratio of I_{537}/I_{476}, indicating that changes to the pH in the range of 7–10 did not affect the fluorescence. Thus the fluorescent studies below were carried out at pH 7.2 in HEPES buffer (50 mM).

As mentioned above, Cu(II) quenched the fluorescence of **M2** at 537 nm, thus we hypothesized that probe **S1** could recover the fluorescence after treatment with histidine because of its high-association constants with Cu^{2+}.[16c,d] Indeed, with the addition of histidine (0–80 μM), the fluorescence of probe **S1** (10 μM) at 537 nm recovered along with an unexpected enhancement of fluorescence intensity at 476 nm, as shown in Fig. 2a. We believed that the histidine acting as a chelating ligand binding to Cu(II) might change the mode of Cu-coordination, which subsequently enhanced the ICT effect, as exemplified in PPi or ATP detection by Zn^{2+} fluorophore complexes.[19] Meanwhile, with His added, the pyridine N atom coordinated with the central Cu^{2+} ion, reducing the charge density on the Cu^{2+} ion, which blocked the ligand-to-metal charge transfer (LMCT). Hence, the fluorescence at 537 nm was recovered.[18] Moreover, histidine also induced a 56 nm hypsochromic shift of the absorption of probe **S1** (Fig. 3). Accordingly, the affinity constant (K_a) of **S1** towards Cu(II) was measured as $K_a = 3.7 \times 10^5$ (Fig S2†). The color of the solution changed from brownish red to light green with the fluorescence switched from off to on (Fig. 2b). There was an 18-fold and 99-fold increase in fluorescent intensity and quantum yield, respectively, after adding histidine (Table 1). Furthermore, a 1 : 1 stoichiometry between **S1** and histidine was found *via* a standard continuous variation titration (Fig. 4).

Initially it was believed that a ligand dissociation between **M2** and Cu^{2+} occurred upon addition of histidine and the fluorescence would recover,[20] as observed in many other

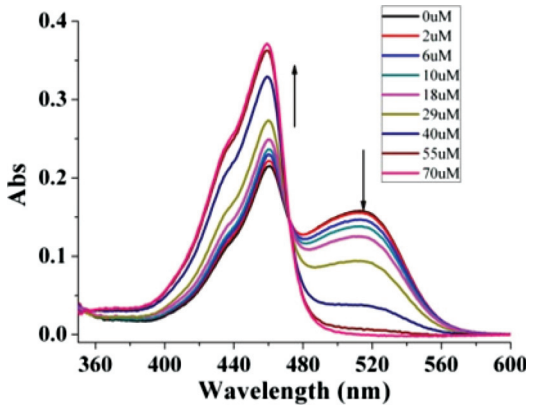


Fig. 3 Absorption spectra of a solution of probe **S1** (10 μM) in the presence of increasing His concentration (0, 2, 6, 10, 18, 29, 40, 55 and 70 μM, respectively) in EtOH–buffer solution (40/60, v/v, 50 mM HEPES, pH 7.2).

Table 1 The spectral data of **M2**, probe **S1** and **S1@His** with absorption at 476 nm, fluorescent intensity at 537 nm and fluorescent quantum yield

Compound	Abs	FI (au)	Φ
M2	0.066	717.5	0.285
Probe S1	0.039	39.1	0.003
S1@His	0.063	722.3	0.296

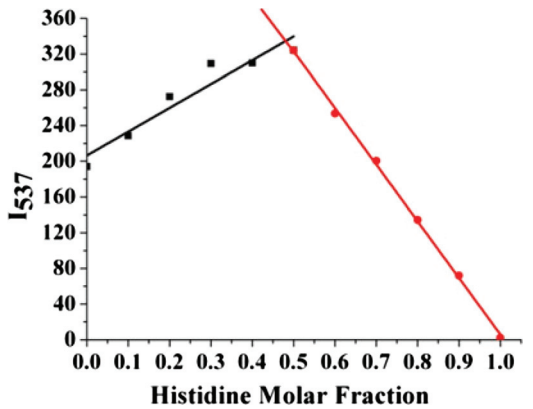


Fig. 4 Job's plot for the probe **S1** complex with His in ethanol–water solution (40 : 60, v/v, 50 mM HEPES buffer, pH 7.2). The total [probe **S1**] + [His] = 10 μM.

metal-complex probes for secondary recognition. The original fluorescence of the **M2** ligand at 537 nm should be recovered and the band at 476 should diminish based on this hypothesis. However, to our surprise, the fluorescence intensity at 476 nm increased as that at 537 increased as well. The unusual phenomenon suggested that a different mechanism in histidine recognition might be in operation. According to the litreature,[21] we assumed that histidine acted as an additional ligand. Therefore, we ran the ESI-MS of the resulting solution by addition of histidine into **S1** solution and found a peak from ESI-MS with *m*/*z* 866.2, which corresponds to [**S1** + ClO_4^- + His + MeOH] (Fig. 5). It clearly indicated that the above hypothesis that the dissociation between **M2** and Cu^{2+} did not occur. Instead, histidine acted as an additional ligand chelating to Cu^{2+} along with

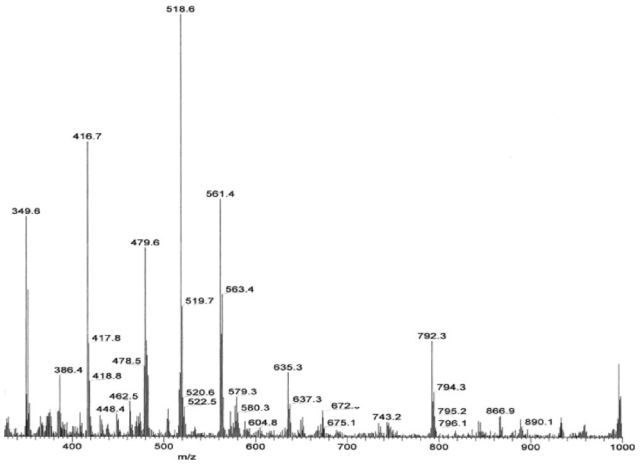


Fig. 5 The ESI-MS assay on detection of histidine with probe **S1**.

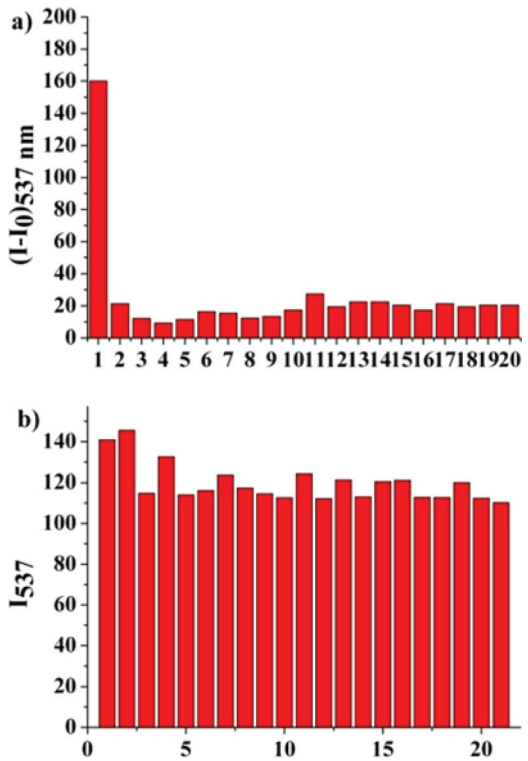


Fig. 6 (a) Fluorescent response of probe **S1** (10 μM) to the selected α-amino acids (His, Trp, Cys, Phe, Gly, Thr, Ile, Pro, Arg, Ser, Met, Lys, Leu, Glu, Ala, Asp, Asn, Gln, Val, Tyr). (b) Fluorescent response of probe **S1** (10 μM) to the competition for other α-amino acids (from left to right: His, all amino acid, Trp, Cys, Phe, Gly, Thr, Ile, Pro, Arg, Ger, Met, Lys, Leu, Glu, Gla, Asp, Asn, Gln, Val, Tyr). Excitation was at 457 nm, and emission was at 537 nm. [Amino acid] = 10 μM.

M2. Meanwhile, the peak of **M2** and [**S1**–Boc] also appeared at 518.6 and 479.6.

To test the selectivity of complex **S1** toward histidine, we titrated the probe **S1** complex solution with other α-amino acids (Trp, Cys, Phe, Gly, Thr, Ile, Pro, Arg, Ger, Met, Lys, Leu, Glu, Gla, Asp, Asn, Gln, Val, Tyr). Though all α- amino acids resulted in an increase in the fluorescence intensity, intensity from histidine was 5-10 fold higher (Fig. 6a). The competition

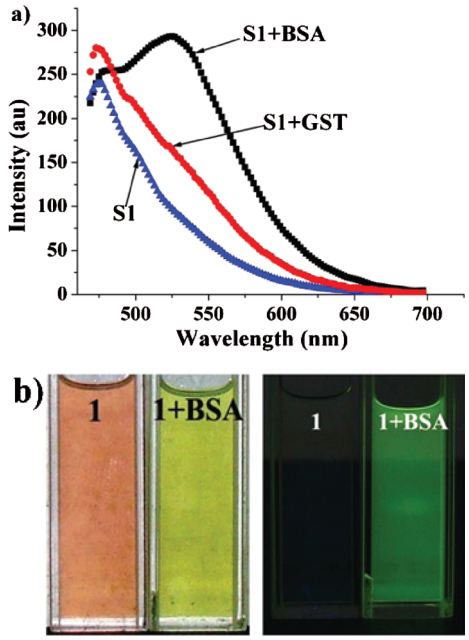


Fig. 7 a) Emission spectra of **S1**(10 μM) in EtOH–buffer solution (40/60, v/v, 50 mM HEPES, pH 7.2) with 0.27 μg BSA (black), 0.27 μg GST (red) and without BSA (blue). b) Photographs of **S1** in the absence and in the presence of BSA.

experiments for the probe **S1** complex were also conducted (Fig. 6b). When the same amount of histidine was added into the probe **S1** solution containing other α-amino acids, the fluorescence enhancement was essentially the same as histidine alone. According to the literature,[22] 5 μM His could induce the 12% increase of the initial fluorescence emission as a reference detection limit. Therefore, probe **S1** could discriminate histidine from other α-amino acids with high selectivity and sensitivity in a buffer solution under neutral pH conditions.

The above results implied that the probe **S1** displayed excellent response to free histidine, and we then investigated if **S1** possessed the same ability in detecting histidine residues on protein surfaces and histidine in live cells. Bovine serum albumin (BSA) is a protein with a relatively high abundance of histidine residues.[16c] It was therefore chosen as a model for this study. In the presence of 5.0 equiv BSA, **S1** exhibited an intense enhancement of the emission at 537 nm as well as a slight enhancement at 476 nm (Fig. 7a). A similar trend was observed using free histidine. Additionally, a GST (Glutathione S-transferases) protein was measured, which only contained 25% histidine residues compared to the BSA protein, and the result, displayed in Fig. 7a, only showed a little enhancement in 537 nm, which also indicated our probe S1 possessed excellent selectivity to histidine residues on protein surfaces. Furthermore, the colorimetric and fluorescent changes were seen (Fig. 7b), which also turned brownish red to light green with an off-on fluorescence.

Subsequently, to further demonstrate the potential practical application of probe **S1** in cell imaging, we applied it to detecting histidine and imaging in living cells. HeLa cell lines were chosen for these studies due to its broad applications in cell studies. As shown in Fig. 8, the normal HeLa cells showed no

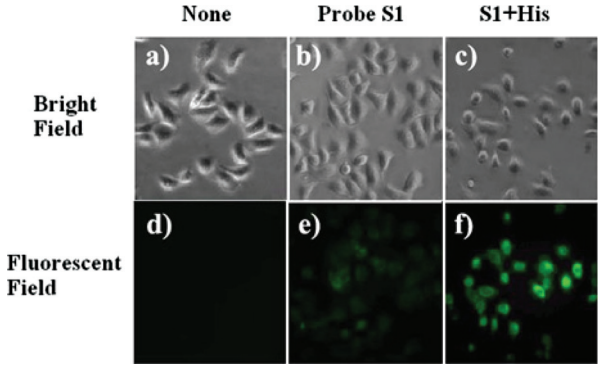


Fig. 8 Fluorescence microphotographs of HeLa cells incubated with none (a) and (d), probe **S1** (b) and (e), and [**S1** + His] (c) and (f). In (c) and (f), histidine was added to the HeLa cells after 30 mins incubation with **S1**. All the cell images were taken after 30 min incubation. (a), (b) and (c) were taken in bright field, and (d), (e) and (f) were taken in fluorescent field in a PBS buffer contain 2% EtOH. The concentration of **S1** and histidine was 10 μM and 100 μM, respectively.

fluorescence. Upon loading of the probe **S1** into the HeLa cell medium, weak blue–green fluorescence emission was observed. After incubating with extra histidine added (100 μM) for several minutes, an enhanced intense green fluorescence was observed. Therefore, **S1** could be used as a useful fluorescent probe for measurement of histidine *in vitro* and in living cells, and also measurement of rich-histidine proteins.

Conclusions

In summary, a novel fluorescent probe **S1** was designed, synthesized and evaluated. Among 20 naturally occurring α-amino acids probed, it specifically responded to histidine with high selectivity and sensitivity under physiological neutral conditions. Furthermore, notably, probe **S1** was demonstrated for detection of histidine residues on proteins and in living cells, indicative of its practical application potential.

Experimental

General methods

All solvents were commercial without further purification. All reactions were carried out under a helium atmosphere with analytic grade solvents, unless noted. Mass spectra were measured on a HP 1100 LC-MS spectrometer. ^{1}H NMR and ^{13}C NMR were measured on a BrukerAV-400 spectrometer with chemical shifts reported in ppm (in CD3OD, or CDCl3; TMS as internal standard). All reactions were monitored by thin-layer chromatography (TLC) using UV-light (254 nm) and Flu-light (365 nm). Silica gel (300–400 mesh) was used for column chromatography. Mass and IR spectral analyses were carried out at the Analysis and Test Center of East China University of Science and Technology (ECUST).

Synthesis of 6-bromo-2-butyl-7-nitro-1*H*-benzo[de]isoquinoline-1,3-(2H)-dione (3). To a solution of 6-bromo-7-nitrobenzo [de]isochromene-1,3-dione **4** (500 mg, 1.55 mmol) in EtOH,

n-butylamine (113 mg, 1.55 mmol) was added, and the mixture was stirred refluxing for 2 h. Then the mixture cooled to room temperature, filtered to afford a yellow needle compound (410 mg) in 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$, 20 °C): δ 8.71 (d, J = 7.6 Hz, 1 H), 8.52 (d, J = 8.0 Hz, 1 H), 8.22 (d, J = 8.0 Hz, 1 H), 7.94 (d, J = 8.0 Hz, 1 H), 4.18 (t, J = 7.6 Hz, 2 H), 1.76–1.68 (m, 2 H), 1.50–1.41 (m, 2 H), 0.99 (dd, J = 7.2 Hz, 7.6 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$, 20 °C): δ 162.79, 162.02, 151.25, 135.96, 132.32, 131.20, 130.53, 125.77, 124.07, 123.54, 122.48, 121.189, 40.74, 30.02, 20.32, 13.79. IR: 2962, 2857, 1707, 1664, 1543, 1347, 1034, 874.

Synthesis of *tert*-butyl(2-((7-bromo-2-butyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[de]isoquinolin-6yl)amino)ethyl)carbamate (2). To a solution of **3** (377 mg, 1.00 mmol) in DMF, N-Boc-ethylenediamine (160 mg, 1.00 mmol) was added and stirred for 6 h at room temperature. Then the mixture was concentrated *in vacuo*. The crude product was purified by column chromatography (silica gel: 100 mL, eluent: DCM) to afford a yellow powder **2** in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$, 20 °C): δ 8.37 (d, J = 8.4 Hz, 1 H), 8.23 (d, J = 8.0 Hz, 1 H), 7.69 (dd, J = 8.0 Hz, 22.4 Hz, 1 H), 6.67 (d, J = 8.4 Hz, 1 H), 5.13 (s, 1 H), 4.12 (dd, J = 7.2 Hz, 7.6 Hz, 2 H), 3.61 (s, 2 H), 3.49 (s, 2 H), 1.73–1.65 (m, 2 H), 1.48 (s, 9 H), 1.46–1.40 (m, 2 H), 0.97 (t, J = 7.2 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$, 20 °C): 163.97, 163.50, 156.10, 150.15, 134.72, 132.23, 131.84, 131.50, 124.59, 122.45, 117.57, 110.16, 105.81, 79.95, 43.75, 40.11, 39.16, 30.17, 28.45, 20.42, 13.88. IR: 3404, 3350, 2959, 1688, 1638, 1310, 1003, 896. ESI-MS 490.1 $[M + H]^+$.

Synthesis of *tert*-butyl(2-((7-(2-pyridine-methylamino)-2-butyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[de]isoquinolin-6-yl)amino) ethyl)carbamate (M2). A solution of **2** (245 mg, 0.50 mmol), 2-aminomethyl-pyridine (540 mg, 5.00 mmol) and ethylene glycol monomethyl ether (30 mL) was refluxed for 7 h. The reaction mixture was cooled to room temperature, and removed *in vacuo*. The crude product was purified by column chromatography (silica gel: 150 mL, eluent: 10% MeOH in DCM) to afford a bright yellow solid in 53% yield.^{1}H NMR (400 MHz, $CDCl_3$, 20 °C): δ 8.72 (s, 1 H), 8.43 (d, J = 8.4 Hz, 1 H), 8.39 (d, J = 8.4 Hz, 1 H), 7.79 (dd, J = 7.2 Hz, 7.6 Hz, 1 H), 7.52 (s, 1 H), 7.41 (d, J = 7.6 Hz, 1 H), 7.31 (dd, J = 5.2 Hz, 20.8 Hz, 1 H), 6.80 (d, J = 8.4 Hz, 1 H), 6.71 (d, J = 8.4 Hz, 1 H), 6.24 (s, 1 H), 5.84 (s, 1 H), 4.66 (s, 2 H), 4.10 (t, J = 7.2 Hz, 2 H), 3.63 (S, 2 H), 3.45 (d, J = 3.6 Hz, 2 H), 1.70–1.67 (m, 2 H), 1.43 (s, 9 H), 1.43–1.40 (m, 2 H), 0.96 (dd, J = 6.8 Hz, 7.2 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$, 20 °C): δ 164.58, 164.54, 156.36, 155.72, 152.52, 151.36, 149.01, 137.13, 133.60, 132.12, 122.78, 122.38, 122.25, 112.27, 111.75, 111.46, 106.80, 106.66, 79.75, 48.58, 45.20, 39.93, 39.72, 30.30, 28.41, 20.46, 13.92. IR: 3301, 2955, 2857, 1682, 1632, 1309. HR-MS (EI): $C_{29}H_{35}N_5O_4$, m/z calculated for 518.2762 $[M + H]^+$, found 518.2761.

Synthesis of probe S1. To a solution of **M2** in MeOH, 1.0 equiv $Cu(ClO_4)_2{\cdot}6H_2O$ was added, and the resulting mixture was stirred overnight. The solvent was removed under vacuum and the obtained sticky solid was recrystallized twice from $MeOH/CH_2Cl_2$ (5/1) to give the complex **S1** as a yellow solid. IR: 3448, 2958, 2857, 1591, 1310. HRMS: $C_{29}H_{34}CuN_5O_4^+$ m/z calcd for 579.1896 $[\mathbf{S1} - H^+]^+$, found 579.1900.

Acknowledgements

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