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J. Org. Chem., Just Accepted Manuscript • DOI: 10.1021/acs.joc.8b02746 • Publication Date (Web): 27 Dec 2018 Downloaded from http://pubs.acs.org on December 31, 2018

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Cell-permeable Fluorogenic Probes for Identification and Imaging Nitroreductases in Live Bacterial Cells

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Abstract Graphic



Abstract

Enzyme-activated fluorogenic probes, which invoke enzymatic catalysis to trigger the generation of fluorescence, provide a versatile platform for monitoring biological processes. The development of fluorogenic probes that can readily penetrate the cell envelopes of bacteria are essential to examine intracellular targets of live bacterial cells. Herein, we present the design, synthesis, properties, and biological applications of two series of fluorogenic probes based on cyanine 5 for identification of bacterial nitroreductase (NTR). The selected fluorogenic probe **3** generates a rapid 10-fold fluorescence response after catalytically reduced by NTR to the intermediate para-aminobenzyl substituted which then underwent a rearrangement elimination reaction. Moreover, probe **3** is cell-permeable for both Gram-positive and Gram-negative bacterial cell envelopes, and is selective for NTR over other biological analytes, thus minimizing the background signal and enabling the real-time intracellular imaging of NTR in live bacterial cells.

Introduction

Fluorescence microscopy is an essential tool for understanding and exploring the mechanisms governing cellular processes at the molecular level¹. Despite the rapid development of techniques to study bacteria, the largely impenetrable cell envelope of these organisms present major challenges in imaging experiments². The ideal probe for bacterial imaging would encompass many favorable properties such as fast response, high brightness, far-red emission and excitation wavelengths, enhanced sensitivity and selectivity for targets, and suitability for biological applications³. The cyanine 5 (Cy 5) fluorophore is a useful fluorescent molecule, being applicable to biological investigations utilizing fluorescence microscopy, owing to its excellent photophysical properties in aqueous media, including outstanding molar extinction coefficient, aqueous solubility and tolerance to physiological pH (pH 3-10), long excitation and emission wavelengths

(620-670 nm), excellent photostability and high quantum yield, and its wide use as a fluorescent tag⁴. However, it has not been widely employed as bacterial fluorogenic probes, which offer the feature of fluorescence turn-on response to the biological analytes without the need to remove or wash off the unreacted probes⁵. One of the reasons for this is probably the lack of a good strategy to improve the bacterial cell envelope permeability and regulate the fluorescence of the Cy 5 fluorophore⁶. Therefore, a novel strategy to overcome bacterial cell envelopes penetration barrier meanwhile regulate the fluorescence of the Cy 5 fluorophore would potentially be applicable to the design of fluorogenic probes for a wide range of intracellular bacterial analytes.

A number of strategies have been reported to develop fluorogenic probes based on cyanine fluorophore, such as fluorescence resonance energy transfer (FRET) mechanism⁷, the modulation of the pi-conjugation⁸ and photoinduced electron transfer⁰. Another common strategy is to chemically cage the fluorophore with a group that can be specially removed by the analyte¹⁰. However, so far these caging strategies are hampered by limited functionalization position of the cyanine chromophore, either at the polyenic methine chain or at the alkyl side chains, and limited caging group variability. In this work, we report two novel series of fluorogenic probes based on Cy 5 with various lipophilic nitroaromatic moieties as caging groups. Instead of functionalizing at the alkyl side chains, the caging groups are decorated at the benzene unit and the methine backbone of Cy 5 core. In addition, the introduction of lipophilic nitroaromatic moieties make the cell-impermeable Cy 5 could penetrate both Gram-positive and Gram-negative bacterial cell envelopes. The selected fluorogenic probes **3** was successfully applied for selective identification and imaging NTR in live bacterial cells (**Figure 1**).



Figure 1. NTR-triggered process of probe 3 in live bacterial cells.

Results and Discussion

Nitroreductases (NTRs), are a family of flavin-containing enzymes, which metabolize nitrosubstituted compounds using the reducing power of nicotinamide adenine dinucleotide (NAD(P)H). NTRs are reported biomarker for tumor and bacterial infection detections¹¹. Previously, we reported an NTR-triggered fluorescent probe by conjugating nitro-imidazole group to the side chain of Cy 5.5, which exhibited fast and sensitive response to NTR, though it is unclear whether this approach can be generalized for other cyanine fluorophores¹². Herein, we studied a novel caging and uncaging strategy by attaching lipophilic nitroaromatic substitutions to Cy 5 fluorophore core structure and designed probes specific for NTR. To improve the fluorescence response to NTR and increase the membrane permeability of probe, we synthesized two series of eight novel probes by introducing different lipophilic nitro groups to R₁ or R₂ positions of Cy 5 carboxyl acid core, respectively (**Figure 2**). By incorporating nitro group, nitrobenzene group, nitroaromatic carbamate moieties into the benzene unit of Cy 5 respectively, the obtained probes **1-4** have an insight into the influence of group lipophilicity and arrangement. Similarly, we obtained probes **5-8** by introducing nitrobenzene group, nitroaromatic carbamate moieties and nitroaromatic ester fragment to the methine backbone of Cy 5, and explored the influence of connecting position of Cy 5 core.



Probe 1-4: R_1 = lipophilic nitroaromatic moieties, R_3 = Methyl Probe 5-8: R_2 = lipophilic nitroaromatic moieties, R_3 = Ethyl

Figure 2. Design strategies of NTR-triggered membrane permeable probes based on cyanine 5.

The heterocyclic ammonium salts (**10a**, **10b**, **10c** and **10d**) were synthesized by quaternization of the corresponding heteroaromatic base 2,3,3-trimethylindolenine (**9a**, **9b** and **9c**) with suitable alkylating agents, including 6-bromohexanoic acid and alkyl halides in *o*-dichlorobenzen or acetonitrile. The three-carbon spacer precursors **11** and **12** were prepared from the commercially available reagents tetramethoxypropane and dibromo-4-oxobut-2-enoic acid, respectively (**Scheme 1**). Probe **1-8** were synthesized as depicted in **Scheme 2**. Three-carbon spacer precursor **11** as starting material on reacting with precursor **10a** gave an intermediate without further purification, which on condensation with precursors **10b**, **10c** furnished asymmetrical cyanine probe **1** and intermediate **13**, respectively. Finally, cyanine intermediate **13** on reacting with different nitroaromatic boric acid reagent (**Scheme S1**) via Suzuki-Miyaura conditions resulted in the formation of probe **2-4** in good yields. In a similar way, cyanine intermediate **14** was prepared from the precursor **12** in good yield. The final probe **5-8** were obtained via Suzuki-Miyaura conditions with corresponding nitroaromatic boric acid reagent (**Scheme S1**). All the obtained products were characterized

by ¹H NMR, ¹³C NMR and HRMS (Supporting information). We expected the selective catalytic reduction

of the probes by NTR would result in an off-on fluorescence response.

Scheme 1. Synthesis of precursors^a



^aReagents and conditions: (i) 6-bromo hexanic acid, bromine ethane, iodomethane, *o*-dichlorobenzen or acetonitrile, 120 °C for 24 h; (ii) aniline, HCl, H₂O, 50 °C for 30 min; (iii) aniline, ethanol, 60 °C for 4 h.

Scheme 2. Synthesis of probe 1 (a), 2-4 (b), 5-8 (c)^{*a*}



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^aReagents and conditions: (i) compound **11** and compound **10a**, AcOH/(Ac)₂O, 130 °C, 2 h, evaporate; (ii) then with compound **10b**, pyridine/AcOH, 120 °C, 2 h; (iii) with compound **10c**, pyridine/AcOH, 120 °C, 2 h; (iv) different nitroaromatic boric acid reagents, Pd(dppf)₂Cl₂, K₂CO₃, 1,4-dioxane, H₂O, 90 °C, 1.5 h; (v) compound **12** and compound **10d**, (Ac)₂O, 60 °C, 4 h; (vi) then with compound **10a**, NaOAc, 50 °C, 2 h; (vii) different nitroaromatic boric acid reagents, Pd(dppf)₂Cl₂, K₂CO₃, 1,4-dioxane, H₂O, 90 °C, 1.5 h.

With these compounds in hand, the fluorescence responses of these probes (probe 1-8) to purified E. coli NTR were first investigated in 0.05 M Tris buffer solution containing 1.5% DMSO (pH 7.4). As depicted in Figure 3 and S1, only probe 1 and 5 showed intense absorption peak with their maximal absorbance at around 645 nm, whereas the other probes exhibited low smooth absorption peak caused by molecular aggregation and poor solubility in above solution. These results indicated that increasing lipophilicity of the probes leads to molecular aggregation and decreased solubility of the probes in aqueous environment. However, a drastic enhancement of the absorption intensity of these probes was observed after incubation with NTR in the presence of NADH. In the fluorescence emission spectra, the fluorescence intensity of the probes was very low except for probe 5 due to its nice aqueous solubility. Moreover, on adding 5 µg/mL NTR in the presence of NADH (500 µM), no significant changes were observed for probe 1, 2, 4, 5 and 6, while obvious fluorescence enhancement were observed for probe 3, 7 and 8 (about 10-fold, 5-fold and 5-fold, respectively). To clarify the reasons for the fluorescence activation differences of probe 1-8 to NTR, we first investigated the reduction response of probe 1-8 to NTR in vitro by HRMS after 1-hour incubation with the enzyme. As shown in Figure S3, probes 2, 4 and 5 could be hardly or not reduced, while the probe 1, 3, 6, 7 and 8 could be reduced and their corresponding amino reduced products were detected after NTR incubation. To clarify the reason for the fluorescence inactivation of probe 1 to NTR, the absorption and fluorescence spectra of chemical synthesized the reduction product of probe 1, product 18, were investigated. As shown in **Figure S1**, product **18** showed low smooth absorption and fluorescence emission in Tris buffer which led to no fluorescence enhancement after probe **1** reduced by NTR. The absorption and fluorescence emission properties of product **19**, NTR reduction product of probe **2**, and product **21**, reduction product of probe **5** and probe **6**, in Tris buffer were also studied. Product **19** exhibited good absorption and fluorescence emission properties, however, only very small amount of product **19** could be detected in the HPLC after 1-hour incubation of probe **2** with NTR (**Figure S4**), which resulted in only a little fluorescence recovery of probe **2**. UV-vis and fluorescence emission intensity of product **21** revealed it was not fluorescent in Tris buffer (**Figure S1i and k**), which might because of the *a*-PET process. Moreover, LC-MS analysis showed strong retention peak of NTR reduction product of probe **3** after 1-hour treatment with NTR (**Figure S3e, f** and **S4b**), and chemical synthesized reduction product of probe **3**, product **20**, shows strong absorption and fluorescence emission intensity (**Figure S1c**). These results indicated that probe **3** has the best NTR detection potentialities.

In order to clarify the fluorescence quenching of probe **3** is induced by photoinduced electron transfer (PET) progress or its aggregations in Tris buffer, we calculated the free-energy changes (ΔG_{eT}) by cyclic voltammetry studies and measured the UV-Vis absorption and fluorescence emission spectra of probes **3** in organic solvent in DMSO and Tris buffer, respectively, and compared with the spectra of product **20**. As shown in **Figure S5**, the nitroaromatic moiety (**25**) act as an electron acceptor for Cyanine fluorophore (**24**) in probe **3**. According Rehm-Weller equation, we got $\Delta G_{eT} < 0$ of probe **3** by cyclic voltammetry, implying *d*-PET process could happen (**Table S1**). Next, probe **3** displayed stronger absorption and fluorescence intensities in DMSO than in Tris buffer, which reveals that probe **3** has a typical aggregation-caused quenching (ACQ) effect in Tris buffer. Moreover, Probe **3** and product **20** exhibited similar absorbance spectra in DMSO; however, the fluorescence intensity of probe **3** showed 15% lower than product **20**.

Therefore, we inferred that ACQ effect and d-PET process quenches probe 3 emission fluorescence synergistically, and the ACQ effect is the major cause for fluorescence quench of probe 3. Furthermore, the primary amine of product 20 is considered to be an electron rich group which may quench fluorescence by a-PET process and protonation could prevents this electron transfer. To verify whether the product 20 has an a-PET process or not, ΔG_{eT} of product 20 and UV-vis and fluorescence emission spectra of product 20 in DMSO and different pH buffer were estimated. The result shows $\Delta G_{eT} > 0$, indicating the PET process from primary amine to the excited Cy chromophore was unlikely happened. As shown in Figure S6, product 20 showed intense absorption peak and emission intensity in DMSO. This result implied unprotonated product 20 itself has excellent fluorescence properties. Moreover, compared with probe 3, product 20 displayed significant absorption and fluorescence emission in Tris buffer which indicated that product 20 has improving solubility in Tris buffer. We evaluated the pH dependence of the fluorescence emission intensity of product 20 in PBS (pH 6.0-8.0) and Tris buffer (pH 7.0-9.0), respectively. Product 20 exhibited significant and similar fluorescence in PBS with different pH values (pH 6.0-8.0) (Figure S7). And in tris buffer (pH 7.0-9.0), the fluorescence intensity decreases slightly with the increase of pH values (Figure 4a). These results indicated that protonation of amino group did not significantly enhance the fluorescence intensity and the primary amino group could not transfer an electron to the excited cyanine chromophore and quench its fluorescence.





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probe **3** in pH 7.4 Tris buffer (control); (ii) (blue line): the system (i) + 500 μ M NADH (another control); (iii) (red line): the system (ii) + NTR (5 μ g/mL); (iv) (purple line): product **20** in pH 7.4 Tris buffer. Spectra were measured after reaction of the probe with NTR for 1 h at 37 °C in 0.05 M Tris buffer solution containing 1.5% DMSO (pH 7.4).

Furthermore, the reduction conditions in terms of pH value and temperature of probe **3** by NTR also revealed that the probe can work effectively under the physiological condition (pH 7.4, 37 °C) (**Figure 4a** and **4b**). Subsequently under the optimized conditions, the fluorescence responses of probe **3** to varied concentrations (1.0-5.0 µg/mL) of NTR were investigated. As depicted in **Figure 4c**, a gradual increase in fluorescence intensity was observed with increase in the NTR concentrations, and good linearity between the fluorescence intensity and the NTR concentrations in the range of 1.0-5.0 µg/mL was observed, with an equation of F = 344.7 C (µg/mL) + 180.0 (R = 0.9903) (**Figure 4d**). The detection limit of the probe for NTR was determined to be 32.9 ng/mL.



Figure 4. Effects of (a) pH and (b) temperature on the fluorescence of probe 3 (5 μ M) reacting with NTR.

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Red line: probe **3** (5 μ M) + NTR (5 μ g/mL) + NADH (500 μ M) in 0.05 M Tris buffer for 1 h at 37 °C; black line: probe **3** (5 μ M) + NADH (500 μ M) in 0.05 M Tris buffer for 1 h at 37 °C; blue line: product **20** (5 μ M) in 0.05 M Tris buffer. (c) Fluorescence emission spectra of probe **3** (5 μ M) reacting with different concentrations of NTR (1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 μ g/mL) in the presence of 500 μ M NADH at 37 °C for 1 h. (d) Linear correlation between the concentration of NTR and the fluorescence intensity of the reaction mixture, data shown as mean ± SD (n = 3).

In order to further study the specificity of probe **3** to NTR, we examined various potential biorelevant interferents, such as reductive endogenous thiols (Cys, DTT, GSH and homocysteine), arginine (Arg), ascorbic acid (Vc), glucose, reactive oxygen species (H_2O_2 , CIO⁻), and inorganic salts (MgCl₂, CaCl₂, KCl and NaCl). As can be seen from **Figure 5a**, when these potential interfering species were added to probe **3** in the presence of 500 μ M NADH, no obvious fluorescence enhancement was observed, including reductive endogenous thiols at a high concentration. Remarkably, when NTR (5 μ g/mL) was added, the significant fluorescence enhancement was observed as before. These results demonstrated that probe **3** shows excellent specificity for NTR over other species and hence can be used for NTR detection.



Figure 5. (a) Fluorescence responses of probe **3** (5 μ M) to various species: NTR (5 μ g/mL), Cys (1 mM), DTT (1 mM), GSH (1 mM), Hcy (1 mM), Arg (1 mM), Vc (1 mM), glucose (10 mM), ClO⁻ (10 μ M), H₂O₂ (10 μ M), MgCl₂ (2.5 mM), CaCl₂ (2.5 mM), KCl (10 mM) and NaCl (10 mM). (b) Fluorescence emission spectra of probe **3** (5 μ M) in the different reaction systems. a: probe **3** in pH 7.4 Tris buffer (control); b: the system a + 500 μ M

NADH (another control); c: the system b + NTR (5 μ g/mL); d: the system c + dicoumarin (2.5 μ M); e: the system c + dicoumarin (5.0 μ M). All spectra were acquired in 0.05 M Tris buffer solution containing 1.5% DMSO (pH 7.4) for 1 h at 37 °C. $\lambda_{ex}/\lambda_{em} = 620/657$ nm.

Furthermore, an enzyme inhibition experiment also proved the fluorescence enhancement was caused by NTR. As shown in Figure 5b, when probe 3 was pre-treated with dicoumarin, a common inhibitor of NTR¹⁴, and then mixed with NTR, the fluorescence intensity was much lower than that without dicoumarin. Moreover, with the increase of dicoumarin concentration, the fluorescence intensity of the mixture of probe 3 and NTR decreased progressively. These results confirmed that the NTR activity was effectively inhibited by dicoumarin and thus the fluorescence off-on response of probe 3 to NTR indeed derives from the enzyme-catalyzed reduction reaction. Moreover, before using probe 3 for its potential applications in bioimaging, time-dependent fluorescence intensity test of probe 3 in vitro was estimated to further understand cellular uptake and substrate reduction. E. coli cells were firstly treated with 10 µM of probe 3 at 37 °C. After incubation for 1 h, the cells were washed three times with Tris buffer, and then continue incubating in Tris buffer for 0-24 h. As shown in Figure S8, prolonging the incubation time led to a progressive increase in fluorescence intensity, confirming that substrate reduction is the key step in the appearance of fluorescence. Further cytotoxic properties were also tested against Hela cells by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays. As seen from Figure S9, there was no obvious cytotoxicity when cells were incubated with probe 3 for 24 h at a concentration of 50 µM.

Having verified the high selectivity of probe **3** for sensing NTR activity *in vitro*, we studied the ability of the probe to detect the NTR in living bacterial pathogens, containing two Gram-positive species, *S. aureus* and Methicillin-resistant *S. aureus* (MRSA), and two Gram-negative species, *K. pneumoniae* and *E. coli*. First, probe **3**

and Cy 5 carboxyl acid (10 µM) (Figure S10) were incubated with four bacterial strains in Tris buffer (0.05 M, pH 7.4) at 37 °C for 1 h, respectively. Then the nucleoid was stained with Hoechst 33258 and the bacteria were imaged by confocal fluorescence microscopy. As shown in Figure 6a, c, e and g, all the four tested bacterial strains incubated with Cy 5 control showed very weak fluorescence signals exciting at 633nm, indicating that Cy 5 carboxyl acid cannot penetrate the tested bacterial cells. However, all the bacteria exhibited bright fluorescent signal after treated with probe 3, which demonstrated that probe 3 was readily taken up and activated by NTRs in live bacterial cells. In addition, the quantification of fluorescence intensity in the bacteria revealed that pre-treatment of bacterial cells with the inhibitor dicoumarin had a significant effect on the reduction of probe 3 (Figure 6b, d, f and h). The fluorescence intensity of these four bacterial strains decreased by 50 % to 60 % in the presence of dicoumarin (Figure 6e), suggesting that the probe 3 was largely activated by NTR intracellularly after internalization.





Figure 6. Confocal microscopy images of *E. coli* (a and b), *S. aureus* (c and d), MRSA (e and f) and *K. pneumoniae* (g and h) incubated with probe **3** and Cy 5 carboxyl acid (10 μ M) in the absence or presence of NTR inhibitor (dicoumarin (0.1 mM)), respectively. DNA was stained with Hoechst 33258 (blue), red: probe and Cy 5 signals. (e) Quantification of fluorescence intensity of probe **3** incubated with *E. coli*, *S. aureus*, MRSA, and *K. pneumoniae* in the absence (red bars) or presence (green bars) of NTR inhibitor, respectively. Bars represent mean values \pm SD (n = 5). Hoechst signal (blue): $\lambda_{ex} = 405$ nm and $\lambda_{em} = 460 \pm 30$ nm; probe and Cy 5 signal (red): $\lambda_{ex} = 633$ nm and $\lambda_{em} = 700 \pm 50$ nm. Scale bar = 10 μ m.

Conclusion

Live-cell fluorescence microscopy of bacteria has allowed researchers to understand many fundamental biological processes. The low permeability of the bacterial cell wall and low target-selectivity have been identified as two major challenging barriers that prevent the visualization of the molecular events inside live bacteria by fluorescent probes. Herein, we present the design and synthesis of two novel series of NTR-triggered fluorogenic probes based on Cy 5 with various lipophilic nitroaromatic moieties as caging groups decorating at the benzene unit or at the methine backbone of Cy 5 core. The introduction of lipophilic nitroaromatic moieties make the cell-impermeable Cy 5 could penetrate both Gram-positive and Gram-negative bacterial cell envelopes. The selected fluorogenic probe **3** demonstrates favorable

characteristics in terms of high specificity, high sensitivity, rapid response to NTR over other biological analytes, thus minimizing the background signal and enabling the real-time intracellular identification and imaging of NTR in live bacterial cells.

Experimental Section

General methods

All the chemicals were purchased from J&K, Energy Chemical or Innochem. Commercially available reagents were used without further purification. Nitroreductase (≥100 units/mg) from *Escherichia coli* and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma-Aldrich. Four bacterial strains (*Escherichia coli* (*E. coli*) (ATCC 25922), *Staphylococcus aureus* (*S. aureus*) (ATCC 29213), *Klebsiella pneumoniae* (*K. pneumoniae*) (ATCC 700603), Methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC 33592)) were purchased from American Type Culture Collection (ATCC), USA. Fluorescence emission spectra and full wavelength absorption spectra were performed on Tecan SparkTM 10M Multimode Microplate Reader. OD values were recorded in a 10 mm path quartz cell on a Metash UV-5100B spectrometer. Confocal laser scanning microscope imaging were conducted with ZEISS LSM 710 Confocal Microscope. All ¹H NMR spectra were recorded at 400 MHz. ¹³C NMR spectra (proton-decoupled carbon data) were recorded at 100 MHz, 125 MHz or 150 MHz, respectively. HRMS was measured with Thermo LCQ Deca XP Max mass spectrometer for ESI and Ion trap.

The Reaction and NMR Data

2,3,3-trimethyl-5-iodo-3*H***-indole (9c).** The 4-iodine phenylhydrazine (511 mg , 2.18 mmol) and 3-methyl-2-butanone (320 mg, 3.72 mmol) were added to the EtOH (20 mL) and added H_2SO_4 (32 µL), the mixture was stirred and refluxed for 4 h. The solution was cooled down to rt and added Na_2CO_3 (pH = 7.0). The mixture was poured into water (20 mL), and extracted with DCM (2 × 20 mL). The combined organic

layer was washed with water (2 × 20 mL), and dried over Na₂SO₄. The solvent was evaporated under reduced pressure to give red solid 607 mg, yield 97 %. Mp 130 – 132 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.81 (d, *J* = 2.0 Hz, H, -Ar), 7.61 (dd, *J*₁ = 8.0 Hz, *J*₂ = 1.6 Hz, H, -Ar), 7.24 (d, *J* = 8.0 Hz, H, -Ar), 2.20 – 2.16 (m, 3H, -CH₃), 1.23 (s, 6H, -CH₃). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 189.0, 153.7, 149.2, 136.6, 131.1, 120.0, 90.7, 54.2, 22.7, 15.5. HRMS (*m/z*) (M+H): calcd. for C₁₁H₁₃NI 286.0087, found 286.0079.

1-(5-carboxypentyl)-2,3,3-trimethyl-3*H*-indolium bromide (10a). The compound 9a (10 g, 62.89 mmol) and 6-bromo hexanic acid (18 g, 92.31 mmol) were added to the *o*-dichlorobenzen (50 mL) and stirred at 120 °C for 24 h. After the reaction was complete, the solution was cooled down to rt and the product was washed by ether. After high-temperature drying to give red solid 14 g, yield 63 %. Mp 132 – 134 °C. ¹H NMR (400 MHz, DMSO- d_6) δ 8.02 – 8.00 (m, H, -Ar), 7.88 – 7.85 (m, H, -Ar), 7.62 – 7.60 (m, 2H, -Ar), 4.49 (t, *J* = 7.6 Hz, 2H, -CH₂-), 2.88 (s, 3H, -CH₃), 2.22 (t, *J* = 6.8 Hz, 2H, -CH₂-), 1.89 – 1.81 (m, 2H, -CH₂-), 1.58 – 1.51 (m, 8H, -CH₂-, -CH₃), 1.47 – 1.41 (m, 2H, -CH₂-). ¹³C NMR (125 MHz, DMSO- d_6) δ 197.1, 174.8, 142.4, 141.6, 129.9, 129.4, 124.1, 116.1, 54.7, 48.1, 34.0, 27.5, 26.0, 24.6, 22.6, 14.8. HRMS (*m*/*z*) (M⁺): calcd. for C₁₇H₂₄O₂N⁺ 274.1802, found 274.1807.

1,2,3,3-tetramethyl-5-nitro-3*H***-indolium iodide (10b).** A mixture of compound **9b** (100 mg, 0.49 mmol) and CH₃I (105 mg, 0.74 mmol) was stirred with acetonitrile (3 mL) in sealing tube at 95 °C for 12 h and the solution was cooled down to rt and the solvent was evaporated under reduced pressure. Then the product was washed by ether, after high-temperature drying to give red solid 89 mg, yield 51 %. Mp 120 – 122 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.70 (s, 1H, -Ar), 8.55 (d, J = 6.4 Hz, 1H, -Ar), 8.11 (d, J = 6.8 Hz, 1H, -Ar), 4.14 (s, 3H, -CH₃), 1.70 (s, 9H, -CH₃). HRMS (m/z) (M⁺): calcd. for C₁₂H₁₅O₂N₂⁺ 219.1128, found 219.1130.

1,2,3,3-tetramethyl-5-iodo-3H-indolium iodide (10c). Following the same procedure for synthesis of

compound **10b** and using compound **9c** (330mg, 1.16 mmol), **10c** was synthesized. The product was washed by ether, after high-temperature drying to give red solid 442 mg, yield 81 %. Mp 108 – 110 °C. ¹H NMR (400 MHz, DMSO- d_6) δ 8.29 (s, H, -Ar), 7.99 (d, J = 7.6 Hz, H, -Ar), 7.72 (d, J = 7.6 Hz, H, -Ar), 3.94 (s, 3H, -CH₃), 2.75 (s, 3H, -CH₃), 1.52 (s, 6H, -CH₃). ¹³C NMR (125 MHz, DMSO- d_6) δ 189.0, 153.7, 149.2, 136.6, 131.1, 122.0, 90.7, 54.2, 22.7, 15.5. HRMS (*m*/*z*) (M⁺): calcd. for C₁₂H₁₅NI⁺ 300.0244, found 300.0245.

1-ethyl-2,3,3-trimethyl-3*H***-indolium bromide (10d).** Following the same procedure for synthesis of compound **10a** and using compound **9a** (10 g, 62.89 mmol) and bromine ethane (13.7 g, 12.57 mmol), **10d** was synthesized. After high-temperature drying to give light purple solid 13 g, yield 77 %. Mp 120 – 122 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.03 – 8.00 (m, H, -Ar), 7.88 – 7.86 (m, H, -Ar), 7.62 – 7.60 (m, 2H, -Ar), 4.56 – 4.51 (m, 2H, -CH₂-), 2.88 (s, 3H, -CH₃), 1.54 (s, 6H, -CH₃), 1.45 (t, *J* = 7.6 Hz, 3H, -CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 196.7, 142.5, 141.3, 129.9, 129.5, 124.1, 115.9, 54.7, 43.7, 22.5, 14.7, 13.3. HRMS (*m/z*) (M⁺): calcd. for C₁₃H₁₈N⁺ 188.1434, found 188.1441.

N-((1*E*, 2*E*)-3-(phenylamino)allylidene)benzenaminium chloride (11). Tetramethoxypropane (4.9 g, 29.88 mmol) and hydrochloric acid (4.25 mL) were added to distilled water (90 mL), and stirred at 50 °C. Then the solution of aniline (5.6 g, 60.22 mmol), hydrochloric acid (5.0 mL) and distilled water (70 mL) was added dropwise to the reaction and continued to stir at 50 °C for 2 h. After cooling, filtered, dried and got solid 5.63 g, yield 73 %. Mp 118 – 120 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.74 (s, 2H, -N*H*-), 8.98 (d, *J* = 11.2 Hz, 2H, -Ar), 7.45 (d, *J* = 4.4 Hz, 8H, -Ar), 7.25 – 7.21 (m, 2H, -C*H*-), 6.54 (t, *J* = 11.6 Hz, 1H, -C*H*-). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 159.0, 139.2, 130.3, 126.3, 117.9, 99.2. HRMS (*m/z*) (M⁺): calcd. for C₁₅H₁₅N₂⁺ 223.1230, found 223.1240.

N-((1E, 2Z)-2-bromo-3-(phenylamino)allylidene)benzenaminium bromide (12). The aniline (0.73 g, 7.84

mmol) was added to EtOH (20 mL) and stirred. The dibromo-4-oxobut-2-enoic acid (1.0 g, 3.89 mmol) was added to EtOH (20 mL), and were added dropwise to the solution of aniline at 0 °C. Then continued to stir at 60 °C for 4 h. After cooling, filtered, dried and got solid 0.95 g, yield 81 %. Mp 135 – 137 °C. ¹H NMR (400 MHz, DMSO-*d*6) δ 9.51 (s, 2H, -Ar), 7.69 (d, *J* = 8.0 Hz, 4H, -Ar), 7.50 (t, *J* = 8.0 Hz, 4H, -Ar), 7.36 – 7.31 (m, 2H, -CH-). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 158.1, 139.1, 132.6, 130.3, 128.3, 127.5, 123.4, 120.1, 89.8. HRMS (*m/z*) (M⁺): calcd. for C₁₅H₁₄BrN₂⁺ 301.0335, found 301.0344.

2-((1E, 3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-5-iodo-1,3,3trimethyl-3H-indolium iodide (13). The compound 11 (1.2 g, 4.64 mmol) and compound 10a (1.6 g, 4.52 mmol) were added to the acetic (10 mL) acetic anhydride (10 mL) and the mixture was stirred and refluxed for 2 h. The solution was cooled down to rt and the solvent was evaporated under reduced pressure. Then acetic (10 mL), pyridine (10 mL) and compound 10c (2 g, 4.68 mmol) were stirred at 120 °C for 2 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM : MeOH = 20 : 1) to give deep blue solid 2.1 g, yield 61 %. Mp 156 – 157 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.32 - 8.18 (m, 2H, -CH-), 7.77 (d, J = 1.2 Hz, 1H, -Ar), 7.71 - 7.68 (m, 1H, -Ar), 7.51 (d, J = 7.2 Hz, 1H, -Ar), 7.43 (t, J = 8.4 Hz, 1H, -Ar), 7.35 (d, J = 8.0 Hz, 1H, -Ar), 7.29 (t, J = 7.2 Hz, 1H, -Ar), 7.05 (d, J = 8.4 Hz, 1H, -Ar), 6.68 (t, J = 12.4 Hz, 1H, -CH-), 6.39 (d, J = 13.6 Hz, 1H, -CH-), 6.20 (d, J = 13.6 Hz, 1H, -CH-), 4.17 (t, J = 7.2 Hz, 2H, -CH₂-), 3.56 (s, 3H, -CH₃), 2.32 (t, J = 7.2 Hz, 2H, -CH₂-), 1.88 – 1.80 (m, 2H, -CH₂-), 1.73 (s, 6H, -CH₃), 1.70 (s, 6H, -CH₃), 1.67 - 1.65 (m, 2H, -CH₂-), 1.55 - 1.49 (m, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 177.3, 175.9, 173.4, 156.3, 154.9, 148.3, 144.7, 144.4, 143.4, 143.0, 138.7, 132.4, 129.9, 127.2, 126.8, 126.4, 123.5, 113.4, 112.5, 105.5, 103.9, 88.4, 51.0, 50.1, 45.1, 34.6, 31.5, 30.8, 28.3, 27.9, 27.3, 25.7. HRMS (*m*/*z*) (M⁺): calcd. for C₃₂H₃₈O₂N₂I⁺ 609.1972, found 609.1959.

2-((1E, 3Z)-3-bromo-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-

ethyl-3,3-dimethyl-3H-indolium bromide (14). Compound 12 (500 mg, 1.31 mmol) and compound 10d
(320 mg, 1.20 mmol) were added to (Ac) ₂ O (20 mL) and the mixture was stirred at 60 °C for 4 h. Then with
compound 10a (425 mg, 1.20 mmol), NaOAc (295 mg, 3.60 mmol), the mixture was stirred at 50 °C for 2h.
After the reaction was complete, the product was purified by silica gel column chromatography (DCM :
MeOH = 20 : 1) to give deep blue solid 437 mg, yield 51 %. Mp 135 – 137 °C. ¹ H NMR (400 MHz,
Methanol- d_4) δ 8.43 (d, $J = 13.2$ Hz, 2H, -CH-), 7.57 (d, $J = 6.8$ Hz, 2H, -Ar), 7.49 – 7.35 (m, 4H, -Ar), 7.21
(d, J = 7.2 Hz, H, -Ar), 7.17 – 7.12 (m, H, -Ar), 6.50 (t, J = 12.8 Hz, 2H, -CH-), 4.30 – 4.20 (m, 4H, -CH ₂ -),
2.34 (t, $J = 6.8$ Hz, 2H, -CH ₂ -), 1.92 (t, $J = 6.8$ Hz, 2H, -CH ₂ -), 1.78 (s, 12H, -CH ₃), 1.75 - 1.72 (m, 2H, 2H, -CH ₂ -))
-CH ₂ -), 1.59 – 1.54 (m, 2H, -CH ₂ -), 1.47 (t, $J = 6.8$ Hz, 3H, -CH ₃). ¹³ C NMR (150 MHz, Methanol- d_4) δ
174.8, 174.5, 149.8, 149.6, 141.8, 141.6, 141.5, 141.4, 130.9, 128.5, 125.6, 122.2, 122.1, 115.6, 111.2,
111.0, 102.3, 101.9, 100.0, 100.0, 65.2, 49.6, 49.6, 43.8, 39.1, 33.3, 26.6, 26.0, 25.9, 24.2, 18.8, 12.6, 10.9.
HRMS (m/z) (M ⁺): calcd. for C ₃₃ H ₄₀ O ₂ N ₂ Br ⁺ 575.2268, found 575.2270.

(4-((((4-nitrobenzyl))oxy)carbonyl)amino)phenyl)boronic acid (15). The 4-nitrophenyl chloroformate (125 mg, 0.58 mmol), 4-amino phenyl boric acid (100 mg, 0.81 mmol) and NaHCO₃ (145 mg, 1.73 mmol) were added to the THF (5 mL) mixture was stirred at rt for 1 h and THF was evaporated to dryness. DCM was added and the mixture was filtered and got white solid, the solid was washed with DCM (30 mL), dried and got white solid 108 mg, yield 59 % (Scheme S1). Mp 155 – 156 °C. ¹H NMR (400 MHz, Methanol-*d*₄) δ 8.22 (d, *J* = 8.8 Hz, 2H, -Ar), 7.63 (d, *J* = 9.6 Hz, 2H, -Ar), 7.55 (d, *J* = 8.4 Hz, 2H, -Ar), 7.44 – 7.37 (m, 2H, -Ar), 5.28 (s, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol-*d*₄) δ 155.2, 149.0, 145.9, 141.5, 135.8, 135.7, 129.3, 124.7, 118.8, 66.1, 49.5. HRMS (*m*/*z*) (M+H): calcd. for C₁₄H₁₃O₆N₂B 316.0867, found 316.0863.

(4-(((((4-nitrobenzyl)oxy)carbonyl)amino)methyl)phenyl)boronic acid (16). Following the same procedure for synthesis of compound 15 and using the 4-nitrophenyl chloroformate (377 mg, 1.75 mmol)

and 4-(aminomethyl)phenylboronic acid (200 mg, 1.46 mmol), compound **16** was synthesized. The product was purified by Pre-TLC (PE : EA = 3 : 1) to give white solid 53 mg, yield 11 % (Scheme **S1**). ¹H NMR (400 MHz, Methanol- d_4) δ 8.21 (d, J = 8.8 Hz, 2H, - Ar), 7.70 (d, J = 9.2 Hz, 1H, -Ar), 7.59 – 7.56 (m, 3H, -Ar), 7.29 – 7.23 (m, 2H, -Ar), 5.22 (s, 2H, -CH₂-), 4.31 (s, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 157.1, 147.5, 144.7, 141.0, 140.5, 133.7, 133.4, 127.6, 126.1, 125.9, 123.1, 114.8, 64.7, 44.1. HRMS (*m/z*) (M+H): calcd. for C₁₅H₁₆O₆N₂B 331.1096, found 331.1093.

(4-(((4-nitrobenzyl)oxy)carbonyl)phenyl)boronic acid (17). The 4-carboxyphenylboronic acid (500 mg, 3.01 mmol), 4-nitrobenzyl bromide (781 mg, 3.62 mmol) and NaHCO₃ (380 mg, 4.52 mmol) were added to the DMF (5 mL) mixture was stirred at rt for 2 h and DMF was evaporated to dryness. The product was purified by silica gel column chromatography (PE : EA = 3 : 1) to give white solid 86 mg, yield 10 % (Scheme **S1**). ¹H NMR (400 MHz, Methanol- d_4) δ 8.27 – 8.25 (m, 2H, -Ar), 8.04 (d, J = 6.8 Hz, 2H, -Ar), 7.73 – 7.69 (m, 4H, -Ar), 5.49 (s, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 166.1, 147.7, 143.7, 133.6, 133.1, 128.1, 123.3, 64.9. HRMS (m/z) (M+H): calcd. for C₁₄H₁₃O₆NB 302.0752, found 302.0738.

2-((1*E*, 3*E*)-5-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-5-nitro-3*H*-indolium iodide (Probe 1). The compound 11 (150 mg, 0.58 mmol) and compound 10a

(205 mg, 0.58 mmol) were added to acetic (3 mL), acetic anhydride (3 mL) and the mixture was stirred and refluxed for 2 h. The solution was cooled down to rt and the solvent was evaporated under reduced pressure. Then acetic (3 mL), pyridine (3 mL) and compound **10b** (200 mg, 0.58 mmol) were stirred at 120 °C for 2 h. After the reaction was complete, the product was purified by silica gel column chromatography (DCM : MeOH = 20 : 1) to give deep blue solid 160 mg, yield 42 %. Mp 158 – 160 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.43 – 8.37 (m, 1H, -CH-), 8.30 – 8.25 (m, 2H, -Ar), 8.22 (t, *J* = 13.2 Hz, 1H, -CH-), 7.61 (d, *J* = 7.2 Hz, 1H, -Ar), 7.52 – 7.50 (m, 2H, -Ar), 7.44 – 7.40 (m, 1H, -Ar), 7.25 (d, *J* = 8.8 Hz, 1H, -Ar), 6.77

 (t, J = 12.0 Hz, 1H, -CH-), 6.65 (d, J = 14.4 Hz, 1H, -CH-), 6.19 (d, J = 12.8 Hz, 1H, -CH-), 4.30 (t, J = 7.2 Hz, 2H, -CH₂-), 3.57 (s, 3H, -CH₃), 2.32 (t, J = 7.2 Hz, 2H, -CH₂-), 1.93 – 1.85 (m, 2H, -CH₂-), 1.78 (s, 6H, -CH₃), 1.76 (s, 6H, -CH₃), 1.72 – 1.66 (m, 2H, -CH₂-), 1.56 – 1.48 (m, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 179.0, 171.1, 157.8, 152.9, 150.2, 144.9, 143.8, 142.9, 130.1, 128.9, 128.3, 126.7, 123.8, 119.0, 113.8, 111.5, 110.1, 108.7, 104.2, 52.0, 46.0, 34.7, 31.1, 30.8, 28.7, 28.1, 27.5, 27.3, 25.7. HRMS (m/z) (M⁺): calcd. for C₃₂H₃₈O₄N₃⁺ 528.2857, found 528.2863.

2-((1E, 3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-5-(4-((((4-nitrobenzyl)oxy)carbonyl)amino)phenyl)-3H-indolium iodide (Probe 2). A mixture of compound 13 (50 mg, 0.07 mmol), compound 15 (26 mg, 0.08 mmol), Pd(dppf)₂Cl₂ (5 mg, 0.007 mmol) and K₂CO₃ (28 mg, 0.20 mmol) was stirred in 1,4-dioxane (5 mL) and H₂O (200 μL) at 90 °C for 1.5 h and then the solvent was evaporated to dryness. The residue was purified by silica gel column chromatography (DCM : MeOH = 20 : 1) to give dark blue solid 38 mg, yield 64 %. Mp 148 – 150 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.28 - 8.25 (m, 4H, -CH-, -Ar), 7.73 (d, J = 2.0 Hz, 1H, -Ar), 7.69 (t, J = 2.4 Hz, 1H, -Ar), 7.67 (d, J = 2.0 Hz, 1H, -Ar), 7.64 (d, J = 2.0 Hz, 1H, -Ar), 7.62 - 7.55 (m, 4H, -Ar), 7.50 - 7.48 (m, 1H, -Ar), 7.67 - 7.48 (m, 1H, -Ar), 7.67 - 7.48 (m, 2H, -Ar), 7.67 - 7.48 (-Ar), 7.43 – 7.39 (m, 1H, -Ar), 7.34 (d, J = 8.4 Hz, 1H, -Ar), 7.30 – 7.24 (m, 2H, -Ar), 6.64 (t, J = 12.4 Hz, 1H, -CH-), 6.31 - 6.27 (m, 2H, -CH-), 5.34 (s, 2H, -CH₂-), 4.11 (t, J = 7.6 Hz, 2H, -CH₂-), 3.65 (s, 3H, -CH₃), 2.32 (t, J = 7.2 Hz, 2H, -CH₂-), 1.83 (t, J = 8.4 Hz, 2H, -CH₂-), 1.78 (s, 6H, -CH₃), 1.73 (s, 6H, -CH₃), 1.70 - 1.66 (m, 2H, -CH₂-), 1.54 - 1.46 (m, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 177.3, 175.1, 174.6, 155.2, 145.9, 143.6, 143.4, 142.7, 139.4, 132.4, 129.9, 129.8, 129.4, 128.4, 128.1, 126.8, 126.3, 124.7, 123.5, 121.5, 120.2, 112.1, 104.7, 104.4, 66.7, 66.2, 50.6, 48.4, 44.8, 34.7, 33.1, 31.8, 31.7, 30.8, 30.5, 28.2, 28.0, 27.9, 27.4, 25.7, 23.8, 20.3, 14.1. HRMS (m/z) (M⁺): calcd. for C₄₆H₄₉O₆N₄⁺ 753.3647, found 753.3623.

2-((1E, 3E)-5-((E)-1-(5-carboxypentyl)-3, 3-dimethylindolin-2-ylidene) penta-1, 3-dien-1-yl)-1, 3, 3-trimeteres and a statistical statis
hyl-5-(4-(((((4-nitrobenzyl)oxy)carbonyl)amino)methyl)phenyl)-3 <i>H</i> -indolium iodide (Probe 3)
Following the same procedure for synthesis of probe 2 and using compound 13 (50 mg, 0.07 mmol) and
compound 16 (27 mg, 0.08 mmol), probe 3 was synthesized. The residue was purified by silica gel column
chromatography (DCM : MeOH = 20 : 1) to give dark blue solid 32 mg, yield 53 %. Mp 135 – 137 °C. ¹ H
NMR (400 MHz, Methanol- d_4) δ 8.29 – 8.22 (m, 4H, -CH-, -Ar), 7.74 (s, 1H, -Ar), 7.67 (d, $J = 8.0$ Hz, 1H
-Ar), 7.61 (d, J = 8.4 Hz, 4H, -Ar), 7.49 (d, J = 6.8 Hz, 1H, -Ar), 7.43 – 7.34 (m, 4H, -Ar), 7.31 – 7.25 (m
2H, -Ar), 6.65 (t, J = 12.4 Hz, 1H, -CH-), 6.32 – 6.27 (m, 2H, -CH-), 5.25 (s, 2H, -CH ₂ -), 4.36 (s, 2H
- CH_2 -), 4.12 (t, $J = 7.2$ Hz, 2H, - CH_2 -), 3.65 (s, 3H, - CH_3), 2.32 (t, $J = 7.2$ Hz, 2H, - CH_2 -), 1.88 – 1.82 (m
2H, -CH ₂ -), 1.78 (s, 6H, -CH ₃), 1.73 (s, 6H, -CH ₃), 1.70 – 1.66 (m, 2H, -CH ₂ -), 1.54 – 1.44 (m, 2H, -CH ₂ -)
¹³ C NMR (150 MHz, Methanol- d_4) δ 177.4, 175.1, 174.8, 158.6, 155.6, 155.3, 146.2, 143.7, 143.6, 143.4
142.7, 140.6, 139.9, 139.4, 129.8, 129.2, 129.0, 128.5, 128.1, 126.8, 126.3, 124.6, 123.5, 121.8, 112.1
104.6, 66.2, 50.7, 50.6, 45.3, 34.7, 31.7, 30.8, 30.5, 30.4, 28.2, 28.0, 27.4, 27.0, 25.7. HRMS (<i>m/z</i>) (M ⁺)
calcd. for $C_{47}H_{51}O_6N_4^+$ 767.3803, found 767.3780.

2-((1*E*, 3*E*)-5-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-5-(4-nitrophenyl)-3*H*-indolium iodide (Probe 4). Following the same procedure for synthesis of probe 2 and using compound 13 (50 mg, 0.07 mmol) and 4-nitrophenylboronic acid (13.6 mg, 0.08 mmol), probe 4 was synthesized. The residue was purified by silica gel column chromatography (DCM : MeOH = 20 : 1) to give dark blue solid 45 mg, yield 71 %. Mp 138 – 140 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.33 – 8.23 (m, 4H, -C*H*-, -Ar), 7.92 (t, *J* = 2.4 Hz, 1H, -Ar), 7.90 (t, *J* = 2.0 Hz, 1H, -Ar), 7.87 (d, *J* = 1.6 Hz, 1H, -Ar), 7.80 – 7.77 (m, 1H, -Ar), 7.53 – 7.51 (m, 1H, -Ar), 7.46 – 7.41 (m, 1H, -Ar), 7.39 – 7.35 (m, 2H, -Ar), 7.32 – 7.28 (m, 1H, -Ar), 6.68 (t, *J* = 12.4 Hz, 1H, -C*H*-), 6.39 (d, *J* = 14.0 Hz, 1H, -C*H*-), 6.26 (d, *J* = 13.6 Hz, 1H, -CH-), 4.17 (t, J = 7.6 Hz, 2H, -CH₂-), 3.63 (s, 3H, -CH₃), 2.32 (t, J = 7.2 Hz, 2H, -CH₂-), 1.89 – 1.83 (m, 2H, -CH₂-), 1.79 (s, 6H, -CH₃), 1.74 (s, 6H, -CH₃), 1.70 – 1.66 (m, 2H, -CH₂-), 1.55 – 1.48 (m, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 177.4, 175.9, 174.2, 156.3, 154.9, 148.3, 148.1, 145.3, 143.5, 143.4, 143.0, 136.5, 130.0, 129.2, 128.7, 127.2, 126.8, 125.2, 123.5, 122.4, 112.6, 112.0, 105.5, 104.3, 51.0, 50.3, 45.1, 34.7, 31.6, 28.3, 28.0, 27.9, 27.4, 25.8. HRMS (m/z) (M⁺): calcd. for C₃₈H₄₂O₄N₃⁺ 604.3170, found 604.3175.

2-((1*E*, 3*Z*)-5-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)-3-(4-nitrophenyl)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3*H*-indolium bromide (Probe 5). Following the same procedure for synthesis of probe 2 and using compound 14 (100 mg, 0.15 mmol), 4-nitrophenylboronic acid (31 mg, 0.18 mmol), probe 5 was synthesized. The residue was purified by silica gel column chromatography (DCM : MeOH = 20: 1) to give dark blue solid 56 mg, yield 53 %. Mp 130 – 132 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.46 (d, *J* = 8.0 Hz, 2H, -Ar), 8.39 (d, *J* = 14.0 Hz, 2H, -C*H*-), 7.59 (d, *J* = 8.0 Hz, 2H, -Ar), 7.52 – 7.49 (m, 2H, -Ar), 7.40 – 7.36 (m, 2H, -Ar), 7.29 – 7.25 (m, 4H, -Ar), 5.68 – 5.62 (m, 2H, -C*H*-), 3.86 – 3.77 (m, 4H, -C*H*₂-), 2.18 (t, *J* = 7.2 Hz, 2H, -C*H*₂-), 1.78 (s, 12H, -C*H*₃), 1.62 – 1.58 (m, 2H, -C*H*₂-), 1.47 – 1.42 (m, 2H, -C*H*₂-), 1.26 (s, 2H, -C*H*₂-), 1.16 (t, *J* = 7.2 Hz, 3H, -C*H*₃). ¹³C NMR (150 MHz, Methanol- d_4) δ 177.7, 175.3, 175.0, 153.8, 149.1, 144.7, 143.3, 142.9, 142.8, 133.0, 130.9, 129.9, 126.8, 125.7, 123.5, 112.3, 112.1, 102.2, 101.6, 71.6, 50.9, 50.8, 44.9, 40.1, 34.9, 30.8, 28.0, 27.8, 27.7, 27.4, 25.6, 12.3. HRMS (*m*/*z*) (M⁺): calcd. for C₃₉H₄₄O₄N₃⁺ 618.3326, found 618.3315.

2-((1E, 3Z)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)-3-(4-((((4-nitrobenzyl)oxy)carbonyl)amino)phenyl)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3H-indolium bromide (Probe 6).
Following the same procedure for synthesis of probe 2 and using compound 14 (100 mg, 0.15 mmol), compound 15 (58 mg, 0.18 mmol), probe 6 was synthesized. The residue was purified by silica gel column

chromatography (DCM : MeOH = 20 : 1) to give dark blue solid 59 mg, yield 50 %. Mp 160 – 162 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.33 (d, J = 14.0 Hz, 2H, -CH-), 8.25 (d, J = 8.8 Hz, 2H, -Ar), 7.71 – 7.67 (m, 4H, -Ar), 7.49 – 7.47 (m, 2H, -Ar), 7.39 – 7.34 (m, 2H, -Ar), 7.26 – 7.21 (m, 6H, -Ar), 5.81 – 5.73 (m, 2H, -CH-), 5.34 (s, 2H, -CH₂-), 3.82 (d, J = 6.8 Hz, 2H, -CH₂-), 3.74 (t, J = 6.8 Hz, 2H, -CH₂-), 2.20 (t, J = 7.2 Hz, 2H, -CH₂-), 1.75 (s, 12H, -CH₃), 1.59 – 1.54 (m, 2H, -CH₂-), 1.50 – 1.41 (m, 2H, -CH₂-), 1.26 – 1.22 (m, 2H, -CH₂-), 1.18 – 1.14 (m, 3H, -CH₃). ¹³C NMR (150 MHz, Methanol- d_4) δ 173.1, 172.9, 153.8, 153.1, 153.0, 147.6, 144.4, 141.9, 141.5, 141.3, 141.2, 138.7, 134.9, 130.5, 128.3, 128.0, 125.0, 125.0, 123.2, 122.1, 122.02, 119.0, 110.5, 110.4, 101.4, 100.8, 64.8, 49.2, 49.1, 43.4, 38.5, 33.8, 29.3, 26.5, 26.3, 26.1, 24.3, 10.8. HRMS (*m*/*z*) (M⁺): calcd. for C₄₇H₅₁O₆N₄⁺767.3803, found 767.3802.

2-((1E, 3*Z*)-5-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)-3-(4-(((((4-nitrobenzyl)oxy)carbonyl)amino)methyl)phenyl)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3*H*-indolium bromide (Probe 7). Following the same procedure for synthesis of probe 2 and using compound 14 (100 mg, 0.15 mmol), compound 16 (60 mg, 0.18 mmol), probe 7 was synthesized. The residue was purified by silica gel column chromatography (DCM : MeOH = 20 : 1) to give dark blue solid 36 mg, yield 54 %. Mp 132 – 134 °C. ¹H NMR (400 MHz, Methanol- d_4) δ 8.33 (d, *J* = 14.0 Hz, 2H, -C*H*-), 8.21 (d, *J* = 8.4 Hz, 2H, -Ar), 7.62 (d, *J* = 8.4 Hz, 2H, -Ar), 7.52 (d, *J* = 8.0 Hz, 2H, -Ar), 7.48 (d, *J* = 7.2 Hz, 2H, -Ar), 7.38 – 7.35 (m, 2H, -Ar), 7.25 (d, *J* = 7.6 Hz, 4H, -Ar), 7.21 (d, *J* = 7.6 Hz, 2H, -Ar), 5.73 (dd, *J*₁ = 14.0 Hz, *J*₂ = 5.2 Hz, 2H, -C*H*-), 5.26 (s, 2H, -C*H*₂-), 4.43 (s, 2H, -C*H*₂-), 3.81 – 3.68 (m, 4H, -C*H*₂-), 2.21 (t, *J* = 6.8 Hz, 2H, -C*H*₂-), 1.75 (s, 12H, -C*H*₃), 1.58 – 1.55 (m, 2H, -C*H*₂-), 1.50 – 1.46 (m, 2H, -C*H*₂-), 1.22 – 1.17 (m, 2H, -C*H*₂-), 1.12 (t, *J* = 6.8 Hz, 3H, -C*H*₃). ¹³C NMR (150 MHz, Methanol- d_4) δ 173.2, 172.9, 157.2, 152.9, 147.5, 144.8, 141.9, 141.5, 141.3, 141.2, 139.4, 134.8, 134.4, 130.1, 128.3, 127.7, 125.0, 123.2, 122.0, 110.6, 110.4, 101.4, 100.8, 64.8, 49.2, 49.1, 43.9, 43.4, 38.4, 33.6, 29.3, 26.5, 26.4, 26.3, 26.0, 24.2, 10.8. HRMS (*m*/*z*) (M⁺): calcd. for C₄₈H₅₃O₆N₄⁺ 781.3960, found 781.3966.

2-((1E, 3Z)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)-3-(4-(((4-nitrobenzyl)oxy)carbonyl)phenyl)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3*H***-indolium bromide (Probe 8).** Following the same procedure for synthesis of probe **2** and using compound **14** (100 mg, 0.15 mmol), compound **17** (55 mg, 0.18 mmol), probe **8** was synthesized. The residue was purified by silica gel column chromatography (DCM : MeOH = 20 : 1) to give dark blue solid 38 mg, yield 30 %. Mp 124 – 126 °C. ¹H NMR (400 MHz, Methanol-*d*₄) δ 8.37 (dd, *J*₁ = 14.0 Hz, *J*₂ = 2.4 Hz, 2H, -C*H*-), 8.31 – 8.27 (m, 4H, - Ar), 7.76 (d, *J* = 8.4 Hz, 2H, -Ar), 7.51 – 7.46 (m, 4H, -Ar), 7.38 – 7.35 (m, 2H, -Ar), 7.28 – 7.22 (m, 4H, -Ar), 5.71 – 5.63 (dd, *J*₁ = 18.8 Hz, *J*₂ = 14.8 Hz, 2H, -C*H*-), 5.54 (s, 2H, -C*H*₂-), 3.84 – 3.72 (m, 4H, -C*H*₂-), 2.13 (t, *J* = 6.4 Hz, 2H, -C*H*₂-), 1.78 (s, 12H, -C*H*₃), 1.58 – 1.54 (m, 2H, -C*H*₂-), 1.43 – 1.39 (m, 2H, -C*H*₂-), 1.29 – 1.26 (m, 2H, -C*H*₂-), 1.14 (t, *J* = 7.2 Hz, 3H, -C*H*₃). ¹³C NMR (150 MHz, Methanol-*d*₄) δ 175.0, 174.7, 167.1, 153.8, 149.3, 145.1, 143.3, 143.0, 142.9, 142.9, 142.7, 134.8, 133.0, 132.4, 132.0, 131.8, 130.9, 130.8, 130.1, 129.8, 126.7, 126.7, 124.8, 123.6, 123.5, 112.2, 112.0, 102.5, 101.9, 66.8, 50.8, 50.7, 44.9, 40.0, 35.1, 30.8, 28.0, 27.8, 27.7, 27.5, 25.6, 20.3, 12.3. HRMS (*m*/z) (M⁺): caled. for C₄₇H₅₀O₆N₃^{*} 752.3694, found 752.3680.

5-amino-2-((1*E*,3*E*)-5-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3, 3-trimethyl-3*H*-indol-1-ium iodide (Product 18). The probe 1 (100 mg, 0.15 mmol), Zn (100 mg, 1.52 mmol) and NH₄Cl (82 mg, 1.52 mmol) were added to the THF (5 mL) and H₂O (1 mL) mixture was stirred at rt for 1 h, THF and H₂O was evaporated to dryness. The product was purified by silica gel column chromatography (DCM : MeOH = 10 : 1) to give dark blue solid 36 mg, yield 38 % (Scheme S2). Mp 142 – 144 °C. ¹H NMR (500 MHz, Methanol- d_4) δ 8.31 – 8.18 (m, 2H, -C*H*-), 7.53 (d, *J* = 6.5 Hz, H, -Ar), 7.46 – 7.43 (m, H, -Ar), 7.40 – 7.32 (m, 5H, -Ar), 6.65 (t, *J* = 12.0 Hz, H, -C*H*-), 6.40 (d, *J* = 14.0 Hz, H, -C*H*-), 6.20 (d, J = 13.0 Hz, H, -CH-), 4.17 (t, J = 7.5 Hz, 2H, -CH₂-), 3.57 (s, 3H, -CH₃), 2.32 (t, J = 6.0 Hz, 2H, -CH₂-), 2.03 (s, 2H, -CH₂-), 1.85 (t, J = 8.0 Hz, 2H, -CH₂-), 1.74 (s, 12H, -CH₃), 1.51 (s, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 177.9, 174.0, 156.7, 155.1, 144.8, 144.0, 143.6, 130.5, 127.5, 124.1, 123.7, 117.7, 113.2, 113.0, 104.7, 51.6, 50.8, 45.8, 35.2, 32.1, 28.9, 28.5, 28.4, 28.0, 26.3. HRMS (*m/z*) (M⁺): calcd. for C₃₂H₄₀O₂N₃⁺ 498.3115, found 498.3115.

5-(4-aminophenyl)-2-((1E,3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien -1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide(Product 19). Following the same procedure for synthesis of probe 2 and using compound 13 (100 mg, 0.14 mmol), (4-aminophenyl)boronic acid hydrochloride (28 mg, 0.16 mmol), product 19 was synthesized. The residue was purified by silica gel column chromatography (DCM : MeOH = 10 : 1) to give dark blue solid 42 mg, yield 44 %. Mp 140 - 142 °C. ¹H NMR (500 MHz, Methanol-d₄) δ 8.27 (dd, J₁ = 24.5 Hz, J₂ = 12.0 Hz, 2H, -CH-), 7.70 (s, 1H, -Ar), 7.63 (d, J = 8.0 Hz, 1H, -Ar), 7.52 (d, J = 7.0 Hz, 1H, -Ar), 7.47 - 7.42 (m, 3H, -Ar), 7.36 (d, J = 8.5 Hz, 1H, -Ar), 7.31 - 7.27 (m, 2H, -Ar), 6.85 (d, J = 8.0 Hz, 2H, -Ar), 6.67 (t, J = 12.0 Hz, 1H, -CH-), 6.36 - 6.28 (dd, $J_1 = 26.0$ Hz, $J_2 = 26.0$ 13.5 Hz, 2H, -CH-), 4.13 (t, J = 7.0 Hz, 2H, -CH₂-), 3.70 (s, 3H, -CH₃), 2.36 (s, 2H, -CH₂-), 1.87 (t, J = 7.5 Hz, 2H, -CH₂-), 1.81 (s, 6H, -CH₃), 1.77 (s, 6H, -CH₃), 1.73 (t, *J* = 7.0 Hz, 2H, -CH₂-), 1.58 – 1.53 (m, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol-d₄) δ 175.3, 174.0, 155.2, 154.8, 148.7, 143.7, 143.4, 142.5, 140.6, 131.2, 129.7, 128.7, 127.4, 126.7, 126.0, 123.4, 120.9, 116.9, 112.2, 111.8, 105.0, 104.0, 50.7, 50.4, 44.7, 31.9, 28.2, 28.1, 27.9, 27.4, 25.9. HRMS (m/z) (M⁺): calcd. for C₃₈H₄₄O₂N₃⁺ 574.3428, found 574.3428. 5-(4-(aminomethyl)phenyl)-2-((1E,3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta -1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide(Product 20). Following the same procedure for synthesis of probe 2 and using compound 13 (100 mg, 0.14 mmol), (4-(aminomethyl)phenyl)boronic acid

hydrochloride (31 mg, 0.16 mmol), product 20 was synthesized. The residue was purified by silica gel

column chromatography (DCM : MeOH = 10 : 1) to give dark blue solid 18 mg, yield 19 %. Mp 160 – 162 °C. ¹H NMR (500 MHz, Methanol- d_4) δ 8.35 – 8.28 (m, 2H, -CH-), 7.82 – 7.80 (m, 3H, -Ar), 7.75 (d, J = 8.0 Hz, 1H, -Ar), 7.60 (d, J = 7.5 Hz, 2H, -Ar), 7.54 (t, J = 7.5 Hz, 1H, -Ar), 7.48 – 7.45 (m, 1H, -Ar), 7.41 (d, J= 8.0 Hz, 1H, -Ar), 7.38 – 7.31 (m, 2H, -Ar), 6.70 (t, J = 12.5 Hz, 1H, -CH-), 6.39 – 6.31 (m, 2H, -CH-), 4.22 (s, 2H, -CH₂-), 4.18 (t, J = 7.0 Hz, 2H, -CH₂-), 3.69 (s, 3H, -CH₃), 2.37 (t, J = 7.0 Hz, 2H, -CH₂-), 1.89 (t, J = 7.5 Hz, 2H, -CH₂-), 1.83 (s, 6H, -CH₃), 1.78 (s, 6H, -CH₃), 1.73 (t, J = 7.0 Hz, 2H, -CH₂-), 1.55 (t, J = 6.5 Hz, 2H, -CH₂-), 1.34 (d, J = 5.0 Hz, 2H, -CH₂-). ¹³C NMR (150 MHz, Methanol- d_4) δ 177.3, 175.3, 174.8, 155.8, 155.10, 144.2, 143.5, 143.4, 142.8, 142.6, 138.5, 133.5, 130.7, 129.8, 128.6, 127.0, 126.5, 123.5, 121.9, 112.3, 112.1, 104.9, 104.4, 50.8, 50.5, 45.0, 44.1, 34.6, 31.6, 28.3, 28.0, 27.9, 27.4, 25.7. HRMS (m/z) (M⁺): calcd. for C₃₉H₄₆O₂N₃⁺ 588.3585, found 588.3588.

2-((1*E***,3***Z***)-3-(4-aminophenyl)-5-((***E***)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien -1-yl)-1-ethyl-3,3-dimethyl-3***H***-indol-1-ium bromide(Product 21). Following the same procedure for synthesis of probe 2 and using compound 14 (100 mg, 0.15 mmol), (4-aminophenyl)boronic acid hydrochloride (34 mg, 0.18 mmol), product 21 was synthesized. The residue was purified by silica gel column chromatography (DCM : MeOH = 10 : 1) to give dark blue solid 30 mg, yield 30 %. Mp 138 – 140 °C. ¹H NMR (500 MHz, Methanol-d_4) \delta 8.34 (d,** *J* **= 16.0 Hz, 2H, -C***H***-), 7.55 – 7.53 (m, 2H, -Ar), 7.45 – 7.41 (m, 2H, -Ar), 7.31 – 7.28 (m, 4H, -Ar), 7.06 (d,** *J* **= 8.5 Hz, 2H, -C***H***-), 6.97 (d,** *J* **= 8.0 Hz, 2H, -Ar), 5.94 (t,** *J* **= 15.0 Hz, 2H, -C***H***-), 3.93 – 3.89 (m, 2H, -C***H***₂-), 3.83 (t,** *J* **= 7.5 Hz, 2H, -C***H***₂-), 2.31 (t,** *J* **= 7.0 Hz, 2H, -C***H***₂-), 1.81 (s, 12H, -C***H***₃), 1.71 – 1.66 (m, 2H, -C***H***₂-), 1.60 (t,** *J* **= 7.5 Hz, 2H, -C***H***₂-), 1.34 (t,** *J* **= 7.5 Hz, 2H, -C***H***₂-), 1.24 (t,** *J* **= 7.0 Hz, 3H, -C***H***₃). ¹³C NMR (150 MHz, Methanol-d_4) \delta 174.3, 174.0, 154.9, 154.8, 150.6, 149.1, 143.5, 143.1, 142.8, 142.6, 141.6, 137.7, 132.0, 129.8, 126.3, 125.3, 123.5, 123.5, 116.8, 111.9, 111.7, 103.2, 102.6, 50.5, 50.5, 544.9, 39.9, 28.1, 27.8, 27.8, 27.7, 26.1, 12.3. HRMS (***m***/z)**

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 (M^+) : calcd. for $C_{39}H_{46}O_2N_3^+$ 588.3585, found 588.3579.

UV-vis absorption and fluorescence spectra of probe 3

The concentration of DMSO stock solution of probe **3** was diluted to 5 μ M in 0.05 M Tris buffer (pH 7.4) with 1.5% DMSO as co-solvent. The UV-visible spectra were recorded using a Tecan SparkTM 10M Multimode Microplate Reader. Wavelength interval: 2.0 nm. Fluorescence spectroscopic studies of probe **3** were performed at the excitation wavelength of 620 nm. Wavelength interval: 2.0 nm.

Cyclic voltammetry

Cyclic voltammetry was performed using a CHI600A analyzer with a scan rate of 100 mV/s at room temperature. The electrolytic cell was a conventional three-electrode cell with a glassy carbon working electrode, an Ag/Ag⁺ (0.01 M AgNO₃) as the reference electrode and Pt wire as the counter electrode. The redox potentials were measured in CH₃CN with 0.1 M of tetra-*n*-butylammonium hexafluorophosphate (*n*-Bu4NPF6) as a supporting electrolyte. ΔG_{eT} values were calculated from the Rehm-Weller equation: ΔG_{eT} = $E_{ox} - E_{red} - \Delta E_{0,0} - wp$ (I), where E_{ox} and E_{red} are oxidation and reduction potentials of electron donor and acceptor, respectively, $\Delta E_{0,0}$ is the excited energy, and wp is the work term for the charge separation and very small.¹⁵ We simplify equation I to equation II: $\Delta G_{eT} = E_{ox} - E_{red} - \Delta E_{0,0}$.

Confocal imaging of bacteria treated with probe 3

E. coli, S. aureus, MRSA, and *K. pneumoniae* cells were cultured for 12 h in respective media at 37 °C. Bacterial strains cultured overnight in respective solution were harvested and washed twice with Tris buffer (pH 7.4). The washed cells were resuspended in Tris buffer (pH 7.4) with an OD₆₀₀ of 0.5-0.7. Then 500 μ L aliquots were treated with 10 μ M of probe **3** or Cy 5 in the presence or absence of the enzyme inhibitor, dicoumarin (0.1 mM). After incubation at 37 °C for 1 h, the cells were washed with Tris buffer (pH 7.4) by centrifugation to remove the unbound reagents and then treated with 20 μ g/mL of Hoechst 33258 at 37 °C

for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads. Fluorescence images were acquired with ZEISS LSM 710 Confocal Microscope (Nikon Eclipse TE2000-E, CFI PlanApochromat VC $63 \times$ oil immersion objective), using a high pressure He-Ne lamp and diode laser for excitation. Hoechst 33258 was excited at 405 nm and its fluorescence was monitored at 430-490 nm, while Cy 5 and probe **3** were excited at 633 nm and the fluorescence was monitored at 650-750 nm.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: ¹H NMR, ¹³C NMR spectra for all compounds and spectroscopic properties of probes.

ACKNOWLEDGMENTS

This work was partially supported by the National Natural Science Foundation of China (NSFC) projects (21778077, 21502236), the Sino-German research project (GZ 1271), the Beijing Nova Program (Z16111000490000), the CAMS Innovation Fund for Medical Sciences (CIFMS 2017-I2M-2-004) and the Fundamental Research Funds for the Central Universities (3332018150).

References

 (a) Abalymov, A. A.; Verkhovskii, R. A.; Novoselova, M. V.; Parakhonskiy, B. V.; Gorin, D. A.; Yashchenok, A. M.; Sukhorukov, G. B. Live-cell imaging by confocal raman and fluorescence microscopy recognizes the crystal structure of calcium carbonate particles in heLa cells. *Biotechnol. J.* 2018, e1800071. (b) Sahl, S. J.; Hell, S. W.; Jakobs, S. Fluorescence nanoscopy in cell biology. *Nat. Rev. Mol. Cell. Biol.* 2017, *18* (11), 685-701. (c) Schlafer, S.; Meyer, R. L. Confocal microscopy imaging of the biofilm matrix. *J. Microbiol. Methods.* 2017, *138*, 50-59. (d) Liu, J. N.; Bu, W.; Shi, J. Chemical design and synthesis of functionalized probes for imaging and treating tumor hypoxia. *Chem. Rev.* **2017**, *117* (9), 6160-6224.

- (a) Han, Y.; Li, M.; Qiu, F.; Zhang, M.; Zhang, Y. H. Cell-permeable organic fluorescent probes for live-cell long-term super-resolution imaging reveal lysosome-mitochondrion interactions. *Nat. Commun.* 2017, *8* (1), 1307. (b) Hirabayashi, K.; Hanaoka, K.; Egawa, T.; Kobayashi, C.; Takahashi, S.; Komatsu, T.; Ueno, T.; Terai, T.; Ikegaya, Y.; Nagano, T.; Urano, Y. Synthesis of practical red fluorescent probe for cytoplasmic calcium ions with greatly improved cell-membrane permeability. *Data. Brief.* 2017, *12*, 351-357. (c) Liang, H.; DeMeester, K. E.; Hou, C. W.; Parent, M. A.; Caplan, J. L.; Grimes, C. L. Metabolic labelling of the carbohydrate core in bacterial peptidoglycan and its applications. *Nat. Commun.* 2017, *8*, 15015. (d) Hsu, Y. P.; Rittichier, J.; Kuru, E.; Yablonowski, J.; Pasciak, E.; Tekkam, S.; Hall, E.; Murphy, B.; Lee, T. K.; Garner, E. C.; Huang, K. C.; Brun, Y. V.; VanNieuwenhze, M. S. Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. *Chem. Sci.* 2017, *8* (9), 6313-6321.
- (a) Jun, Y. W.; Kim, H. R.; Reo, Y. J.; Dai, M.; Ahn, K. H. Addressing the autofluorescence issue in deep tissue imaging by two-photon microscopy: the significance of far-red emitting dyes. *Chem. Sci.* 2017, *8* (11), 7696-7704. (b) Levine, S. R.; Beatty, K. E. Synthesis of a far-red fluorophore and its use as an esterase probe in living cells. *Chem. Commun (Camb).* 2016, *52* (9), 1835-1838. (c) Tallman, K. R.; Levine, S. R.; Beatty, K. E. Profiling esterases in mycobacterium tuberculosis using far-red fluorogenic substrates. *ACS Chem. Biol.* 2016, *11* (7), 1810-1815. (d) Zhang, Y.; Song, K. H.; Tang, S.; Ravelo, L.; Cusido, J.; Sun, C.; Zhang, H. F.; Raymo, F. M. Far-red photoactivatable BODIPYs for the super-resolution imaging of live cells. *J. Am. Chem. Soc.* 2018, *140* (40), 12741-12745. (e) Liu, J.; Guo, X.; Hu, R.; Liu, X.; Wang, S.; Li, S.; Li, Y.; Yang, G. Molecular engineering of aqueous soluble

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triarylboron-compound-based two-photon fluorescent probe for mitochondria H₂S with analyte-induced finite aggregation and excellent membrane permeability. *Anal. Chem.* **2016**, *88* (1), 1052-1057. (f) Xu, J.; Pan, J.; Jiang, X.; Qin, C.; Zeng, L.; Zhang, H.; Zhang, J. F. A mitochondria-targeted ratiometric fluorescent probe for rapid, sensitive and specific detection of biological SO₂ derivatives in living cells. *Biosens. Bioelectron.* **2016**, *77*, 725-732.

- (a) Michie, M. S.; Gotz, R.; Franke, C.; Bowler, M.; Kumari, N.; Magidson, V.; Levitus, M.; Loncarek, J.; Sauer, M.; Schnermann, M. J. Cyanine conformational restraint in the far-red range. *J. Am. Chem. Soc.* 2017, *139* (36), 12406-12409. (b) Briggs, M. S.; Burns, D. D.; Cooper, M. E.; Gregory, S. J. A pH sensitive fluorescent cyanine dye for biological applications. *Chem. Commun (Camb).* 2000, (23), 2323-2324. (c) Widengren, J.; Schwille, P. Characterization of photoinduced isomerization and back-isomerization of the cyanine dye Cy 5 by fluorescence correlation spectroscopy. *J. Phys. Chem. A.* 2000, *104* (27), 6416-6428. (d) Mujumdar, S. R.; Mujumdar, R. B.; Grant, C. M.; Waggoner, A. S. Cyanine-labeling reagents: sulfobenzindocyanine succinimidyl esters. *Bioconjug. Chem.* 1996, *7* (3), 356-362.
- (a) Li, Y.; Yu, H.; Qian, Y.; Hu, J.; Liu, S. Amphiphilic star copolymer-based bimodal fluorogenic/magnetic resonance probes for concomitant bacteria detection and inhibition. *Adv. Mater.* **2014**, *26* (39), 6734-6741. (b) Wu, J.; Zawistowski, A.; Ehrmann, M.; Yi, T.; Schmuck, C. Peptide functionalized polydiacetylene liposomes act as a fluorescent turn-on sensor for bacterial lipopolysaccharide. *J. Am. Chem. Soc.* **2011**, *133* (25), 9720-9723. (c) Sunbul, M.; Jaschke, A. Contact-mediated quenching for RNA imaging in bacteria with a fluorophore-binding aptamer. *Angew. Chem. Int. Ed. Engl.* **2013**, *52* (50), 13401-13404. (d) Feng, G.; Yuan, Y.; Fang, H.; Zhang, R.; Xing, B.; Zhang, G.; Zhang, D.; Liu, B. A light-up probe with aggregation-induced emission characteristics

(AIE) for selective imaging, naked-eye detection and photodynamic killing of Gram-positive bacteria. *Chem. Commun (Camb).* **2015**, *51* (62), 12490-12493.

- Zhang, Q.; Wang, Q.; Xu, S.; Zuo, L.; You, X.; Hu, H. Y., Aminoglycoside-based novel probes for bacterial diagnostic and therapeutic applications. *Chem. Commun (Camb).* 2017, *53* (8), 1366-1369.
- (a) Moroz, P.; Jin, Z.; Sugiyama, Y.; Lara, D.; Razgoniaeva, N.; Yang, M.; Kholmicheva, N.; Khon, D.; Mattoussi, H.; Zamkov, M. Competition of charge and energy transfer processes in donor-acceptor fluorescence pairs: calibrating the spectroscopic ruler. *ACS Nano.* 2018, *12* (6), 5657–5665. (b) Zhang, X. T.; Gu, Z. Y.; Liu, L.; Wang, S.; Xing, G. W. Synthesis and labeling of alpha-(2,9)-trisialic acid with cyanine dyes for imaging of glycan-binding receptors on living cells. *Chem. Commun (Camb).* 2015, *51* (41), 8606-8609. (c) Nicoli, F.; Barth, A.; Bae, W.; Neukirchinger, F.; Crevenna, A. H.; Lamb, D. C.; Liedl, T. Directional photonic wire mediated by homo-forster resonance energy transfer on a DNA origami platform. *ACS Nano.* 2017, *11* (11), 11264-11272.
- (a) Gunasekaran, S.; Hernangomez-Perez, D.; Davydenko, I.; Marder, S.; Evers, F.; Venkataraman, L. Near length-independent conductance in polymethine molecular wires. *Nano. Lett.* 2018, *18* (10), 6387-6391. (b) He, L.; Yang, X.; Xu, K.; Kong, X.; Lin, W. A multi-signal fluorescent probe for simultaneously distinguishing and sequentially sensing cysteine/homocysteine, glutathione, and hydrogen sulfide in living cells. *Chem. Sci.* 2017, *8* (9), 6257-6265. (c) Sha, X. L.; Xiao, J. W.; Yin, D. H.; Sun, R.; Xu, Y. J.; Ge, J. F. Preparation of a photostable tribrachia cyanine dye and its high chemical activity towards hydrosulfide. *Dyes. Pigm.* 2018, *149*, 505-511.
- (a) Xu, K.; Wang, F.; Pan, X.; Liu, R.; Ma, J.; Kong, F.; Tang, B. High selectivity imaging of nitroreductase using a near-infrared fluorescence probe in hypoxic tumor. *Chem. Commun (Camb)*.
 2013, 49 (25), 2554-2556. (b) Li, Y.; Sun, Y.; Li, J.; Su, Q.; Yuan, W.; Dai, Y.; Han, C.; Wang, Q.;

 Feng, W.; Li, F. Ultrasensitive near-infrared fluorescence-enhanced probe for in vivo nitroreductase imaging. J. Am. Chem. Soc. 2015, 137 (19), 6407-6416.

- (a) Xue, C.; Lei, Y.; Zhang, S.; Sha, Y. A cyanine-derived "turn-on" fluorescent probe for imaging nitroreductase in hypoxic tumor cells. *Anal. Methods.* 2015, 7 (24), 10125-10128. (b) Zhu, D.; Xue, L.; Li, G.; Jiang, H. A highly sensitive near-infrared ratiometric fluorescent probe for detecting nitroreductase and cellular imaging. *Sensor. Actuat. B-Chem.* 2016, *222*, 419-424. (c) Shi, Y.; Zhang, S.; Zhang, X. A novel near-infrared fluorescent probe for selectively sensing nitroreductase (NTR) in an aqueous medium. *Analyst.* 2013, *138* (7), 1952-1955. (d) Klotzner, D. P.; Klehs, K.; Heilemann, M.; Heckel, A. A new photoactivatable near-infrared-emitting QCy7 fluorophore for single-molecule super-resolution microscopy. *Chem. Commun (Camb).* 2017, *53* (71), 9874-9877. (e) Chevalier, A.; Zhang, Y.; Khdour, O. M.; Kaye, J. B.; Hecht, S. M. Mitochondrial nitroreductase activity enables selective imaging and therapeutic targeting. *J. Am. Chem. Soc.* 2016, *138* (37), 12009-12012.
- (a) Bae, J.; McNamara, L. E.; Nael, M. A.; Mahdi, F.; Doerksen, R. J.; Bidwell, G. L.; Hammer, N. I.; Jo, S. Nitroreductase-triggered activation of a novel caged fluorescent probe obtained from methylene blue. *Chem. Commun (Camb).* 2015, *51* (64), 12787-12790. (b) Wong, R. H.; Kwong, T.; Yau, K. H.; Au-Yeung, H. Y. Real time detection of live microbes using a highly sensitive bioluminescent nitroreductase probe. *Chem. Commun (Camb).* 2015, *51* (21), 4440-4442. (c) Feng, P.; Zhang, H.; Deng, Q.; Liu, W.; Yang, L.; Li, G.; Chen, G.; Du, L.; Ke, B.; Li, M. Real-time bioluminescence imaging of nitroreductase in mouse model. *Anal. Chem.* 2016, *88* (11), 5610-5614.
- Xu, S.; Wang, Q.; Zhang, Q.; Zhang, L.; Zuo, L.; Jiang, J. D.; Hu, H. Y. Real time detection of ESKAPE pathogens by a nitroreductase-triggered fluorescence turn-on probe. *Chem. Commun (Camb)*.
 2017, 53 (81), 11177-11180.

- (a) He, Y. G.; Shi, S. Y.; Liu, N.; Ding, Y. S.; Yin, J.; Wu, Z. Q. Tetraphenylethene-functionalized conjugated helical poly (phenyl isocyanide) with tunable light emission, assembly morphology, and specific applications. *Macromolecules*. 2015, *49* (1), 48-58. (b) Han, X.; Zhang, J.; Qiao, C. Y.; Zhang, W. M.; Yin, J.; Wu, Z. Q. High-efficiency cell-penetrating helical poly (phenyl isocyanide) chains modified cellular tracer and nanovectors with thiol ratiometric fluorescence imaging performance. *Macromolecules*. 2017, *50* (11), 4114-4125. (c) Fu, B.; Huang, J.; Bai, D.; Xie, Y.; Wang, Y.; Wang, S.; Zhou, X. Label-free detection of pH based on the i-motif using an aggregation-caused quenching strategy. *Chem. Commun (Camb)*. 2015, *51* (95), 16960-16963. (d) Li, K.; Wang, J.; Li, Y.; Si, Y.; He, J.; Meng, X.; Hou, H.; Tang, B. Z. Combining two different strategies to overcome the aggregation caused quenching effect in the design of ratiometric fluorescence chemodosimeters for pH sensing. *Sensor, Actuat. B-Chem*. 2018, *274*, 654-661.
- Koder, R. L.; Miller, A. F. Steady-state kinetic mechanism, stereospecificity, substrate and inhibitor specificity of Enterobacter cloacae nitroreductase. *Biochim. Biophys. Acta*. 1998, 1387 (1-2), 395-405.
- 15. (a) Verma, M.; Chaudhry, A. F.; Fahrni, C. J. Predicting the photoinduced electron transfer thermodynamics in polyfluorinated 1,3,5-triarylpyrazolines based on multiple linear free energy relationships. *Org. Biomol. Chem.* 2009, 7 (8), 1536-1546. (b) Ueno, T.; Urano, Y.; Kojima, H.; Nagano, T. Mechanism-based molecular design of highly selective fluorescence probes for nitrative stress. *J. Am. Chem. Soc.* 2006, *128* (33), 10640-10641. (c) Rehm, D.; Weller, A. Kinetics of Fluorescence Quenching by Electron and H-Atom Transfer. *Israel. J. Chem.* 1970, *8* (2), 259-271.