Supporting information

Highly sensitive water-soluble luminol chemiluminescence and its application in forensic bloodstain detection

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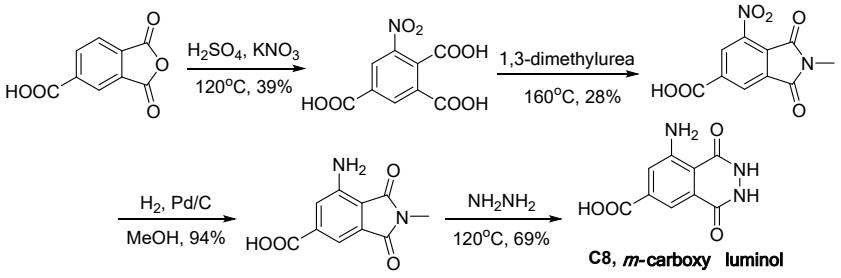
1. Reagents and apparatus

All reagents were commercially available and used without further purification unless indicated otherwise. *m*-Carboxy luminol was initially synthesized by the group of Professor Jacobi von Wangelin according to Ref. [1]. Thin layer chromatographies were carried out on GF254 plates. Flash chromatography was performed with 200-300 mesh silica gels. The deionized water from a Milli-Q Plus system was used throughout the experiments. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer. Chemical shifts (δ) are expressed in ppm, and J-values are given in Hz. The residual solvent protons (^{1}H) or the solvent carbons (^{13}C) were used as internal standards. Data was reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, and m = multiplet), coupling constant (*J* values) in Hz and integration. Luminescence signal for measurements are recorded by a handheld Hygiena™ EnSURE™ ATP Monitoring System. All of the experiments were performed at 25℃.

2. Syntheses of *m*-Carboxy luminol

m-Carboxy luminol was initially synthesized by the group of Professor Jacobi von Wangelin according to Ref. [1]. ^{1}H NMR (400 MHz, D_2O+HNO_3): δ: 7.58 (s, 1H), 7.27 (s, 1H). ^{13}C NMR (100 MHz, D_2O+HNO_3): δ: 174.52, 162.07, 161.24, 148.87, 140.89, 130.56, 117.43, 114.26, 113.91. LC-MS (ESI): m/z = calcd. for $C_9H_7N_3O_4$ (M-H): 220.0; found: 220.1.



Scheme 1. Synthetic route of *m*-Carboxy luminol.

3. photophysical properties

25 mg of solid luminol and its derivative **C8** were measured by a Hitachi F-4700 fluorescence spectrophotometer. According to Figure S1, when The emission at 365 nm excited by UV, the maximum fluorescence emission wavelength of solid luminol (**C1**) was 435.2 nm, and the maximum emission wavelengths were gradually red shifted to 498.6 nm with carboxyl group introduction (Figure S1). As indicated from Fig. 1, **C8** has higher fluorescence intensity than **C1**, and its fluorescence stability is outstanding.

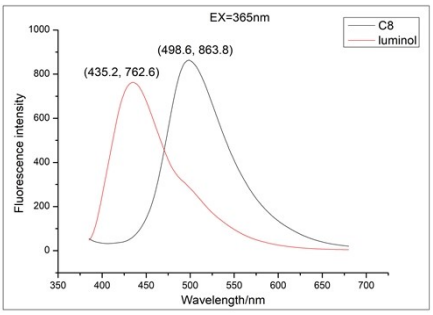


Figure S1: Fluorescence properties of the solid luminol and its derivative **C8** at 365nm excitation wavelength

The UV-Vis absorption spectra of acetonitrile solutions (1×10^{-6} M) of **C1** and **C8** were recorded on HITACHI U-4100 UV-Visible Spectrophotometer. Photophysical studies show that **C8** retains typical luminol characteristics (**Fig. S2**)

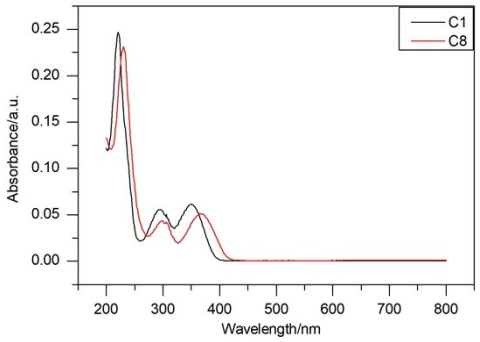


Fig. S2. Absorbance measurements of **C1** and **C8**.

Tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl) buffer (1.0 mL) with different pHs were used as the reaction mediums. The pH effect indicated that **C8** exhibited a decrease in fluorescence intensity over luminol at pH 4.60, 5.76, 6.77, 7.44, 7.94, 8.39, 8.87, 9.55, 10.14. The maximum emission wavelength of **C1** shifted from 425 to 439 nm due to carboxyl group introduction. In addition, the fluorescence intensity of **C1** and **C8** was strongest at pH 4.60. these results indicated that the background intensity of **C8** becomes lower. But, it should be encouraged to search for fluorescence substrates that allow the testing at milder and less hazardous conditions (e.g. a mild pH condition).

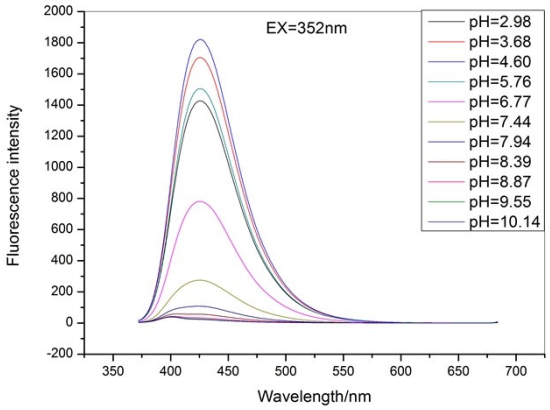


Figure S3: Fluorescence properties of luminol under different pHs

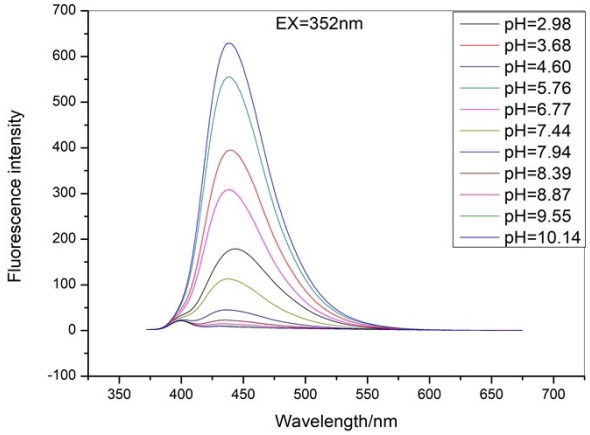


Figure S4: Fluorescence properties of **C8** under different pHs

Tris·HCl buffer (1.0 mL, pH=4.6) was used to prepare reaction solutions. The fluorescence intensity of **C8** (1.0 μM) at 439 nm gradually increased and was obviously augmented by 86% when the added concentration of H_2O_2 was from 0.3 μmol/L to 1.5 μmol/L, Continuing to increase the concentration of H_2O_2 while was no increased significantly in fluorescence intensity.

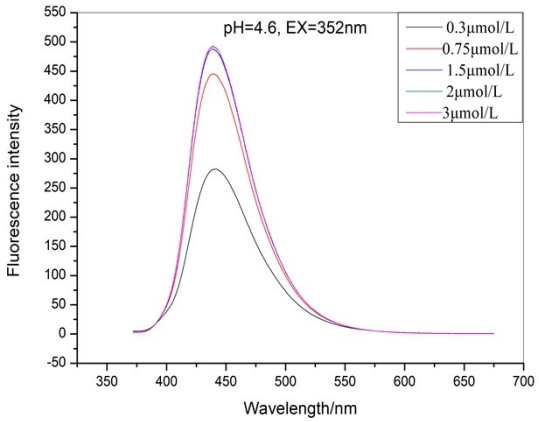


Figure S5: Fluorescence properties of **C8** under different H_2O_2 concentrations

Tris·HCl buffer (1.0 mL, pH=4.6) with different **C8** concentrations were used to prepare reaction solutions. The fluorescence intensity of the **C8** solution was gradually increased when the concentration of **C8** was from 0.1 μmol/L to 5 μmol/L (Figure S6).

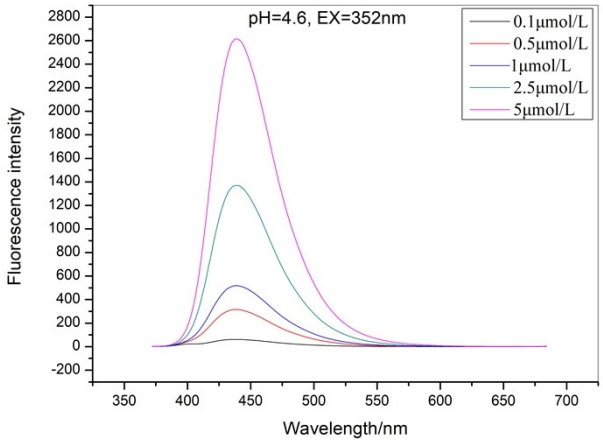


Figure S6: Fluorescence properties at different **C8** concentrations

Human blood was sequentially diluted to a solution of 0.25 μL/L, 2.5 μL/L, 25 μL/L, 125 μL/L, 250 μL/L, 625 μL/L, 1.25 mL/L with deionised water. The test solution consisted of a mixture of luminol derivative **C8** solution（0.1 mL, 10 μmol/L）, Tris-HCl buffer（0.7 mL, pH=4.6）, H_2O_2（0.1mL, 15 μmol/L）and blood solution（0.1 mL）. The final mixture volume was 1 mL, 10 times of dilution of **C8**, H_2O_2 and blood. The fluorescence intensity of **C8** solution increased slightly when the blood concentration was increased from 25 nL/L to 250 nL/L, However, gradually decreased when the blood concentration was increased from 250 nL/L to 125 μL/L.

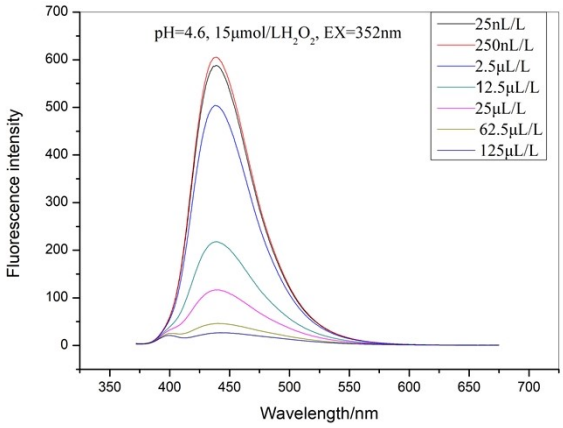


Figure S7: Fluorescence properties of **C8** under different blood concentrations

4. CL properties of luminol derivatives

For a typical test, CL substrate was mixed with hemin followed by the addition of H_2O_2. The CL signals were collected immediately through the luminescence microplate reader with 1-second interval. The test solution consisted of a mixture of luminol solution (2.8 mmol/L, 50 μL), blood (30 μL), H_2O_2 (10 μL) and deionized water (3.9 mL), with seven parallel experiments. According to the above seven groups of experiments, with the increase of K_2CO_3 concentration in the detection solution, the chemiluminescence intensity of luminol aqueous solution was gradually increased, when the

concentration of K_2CO_3 is less than 4.2 mmol/L, the chemiluminescence intensity is very weak and the light intensity is less than 662. When the concentration of K_2CO_3 was increased from 11.2 mmol/L to 14 mmol/L, the maximum chemiluminescence intensity was increased from 13263 to 15412, when the concentration of K_2CO_3 was increased from 14 mmol/L to 36.4mmol/L, the maximum chemiluminescence intensity was increased from 15412 to 16320. It can be seen that when the concentration of K_2CO_3 reached 14 mmol/L, continuing to increase the concentration of luminol, the chemiluminescence intensity of luminol change a little.

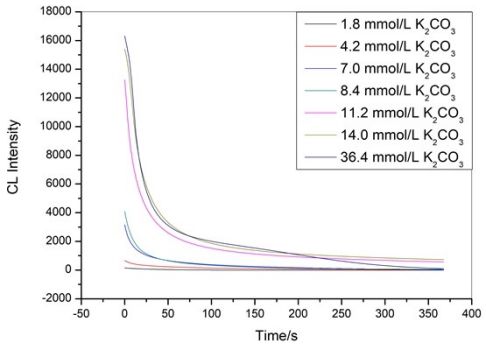


Figure S8: CL properties of luminol under different K_2CO_3 concentration

For a typical test, CL substrate was mixed with hemin followed by the addition of H_2O_2. The CL signals were collected immediately through the luminescence microplate reader with 1-second interval. The test solution consisted of a mixture of **C8** solution (2.8 mmol/L, 50 μL), blood (30 μL), H_2O_2 (10 μL) and deionized water (3.9 mL), with eight parallel experiments. According to the above eight groups of experiments, with the increase of K_2CO_3 concentration in the detection solution, the chemiluminescence intensity of **C8** aqueous solution was gradually increased, when the concentration of K_2CO_3 is less than 1.8 mmol/L, the chemiluminescence intensity is very weak and the light intensity is less than 746. When the concentration of K_2CO_3 was increased from 11.2 mmol/L to 14 mmol/L, the maximum chemiluminescence intensity was increased from 17316 to 17477. It can be seen that when the concentration of K_2CO_3 reached 11.2 mmol/L, continuing to increase the concentration of **C8**, the chemiluminescence intensity of **C8** change a little.

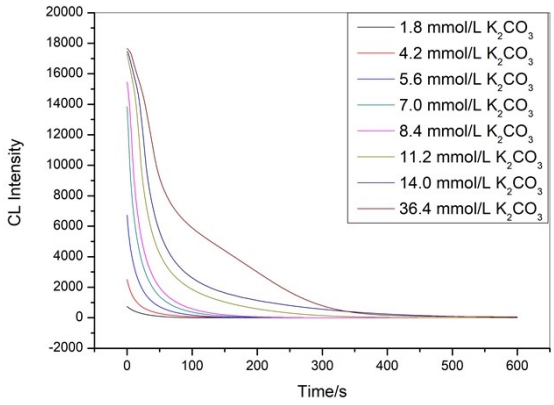


Figure S9: CL properties of **C8** under different K_2CO_3 concentration

The CL duration of **C1** and **C8**

Luminol (**C1**, 100 mg) and *m*-carboxy luminol (**C8**, 100 mg**)** aqueous solution with K_2CO_3 (500 mg) respectively were used as the reaction mediums. **C1** (0.5 mL) and **C8** (0.5 mL) solutions, add deionized water (0.5 mL) and 30% H_2O_2 (30 μL), turn off the light, add blood solution (30 μL) (deionized water: blood =12:1), and quickly record with the camera. we found that C8 exhibited greater CL emitting than luminol. Comparative studies revealed that the CL intensity of luminol was weak after 96 s, while **C8** lasted more than 218 s with twice the duration.

5. Bloodstain detection

Preparation of luminol derivative **C8** storage solution: K_2CO_3 (1.25 g) was dissolved in deionized water (25 mL), **C8** (100 mg) was added, shaken and dissolved, transferred to a brown bottle and protected from light.

Preparation of **C8** working solution: take **C8** (25 mL) storage solution and add 30% H_2O_2 (1.5 mL) to make **C8** working solution.

Preparation of **C1** storage solution: K_2CO_3 (1.25 g) was dissolved in deionized water (25 mL), **C1** (90 mg) was added, shaken and dissolved, transferred to a brown bottle and protected from light.

Preparation of **C1** working solution: take **C1** (25 mL) storage solution and add 30% H_2O_2 (1.5 mL) to make **C1** working solution.

Briefly: the blood was diluted with pure water to reach gradient concentrations of 1/10, 1/100, 1/200, 1/500, 1/1000; the corresponding diluted samples were dripped onto different pieces of 6-mm-diameter filter paper. After it is dried, **C1** and **C8** working solution were sprayed against these samples through a small spray bottle (15 mL), then the CL imaging was taken immediately in dark box using smart phone. the CL imaging reagents containing **C8** show great advantages over luminol in bloodstain imaging. Upon 1000 times dilution, **C8** can still show obvious CL signal, whereas, luminol can no longer perform effective imaging. The result implies that imaging solutions with **C8** have excellent application prospects in criminal investigations.

6. NMR spectra

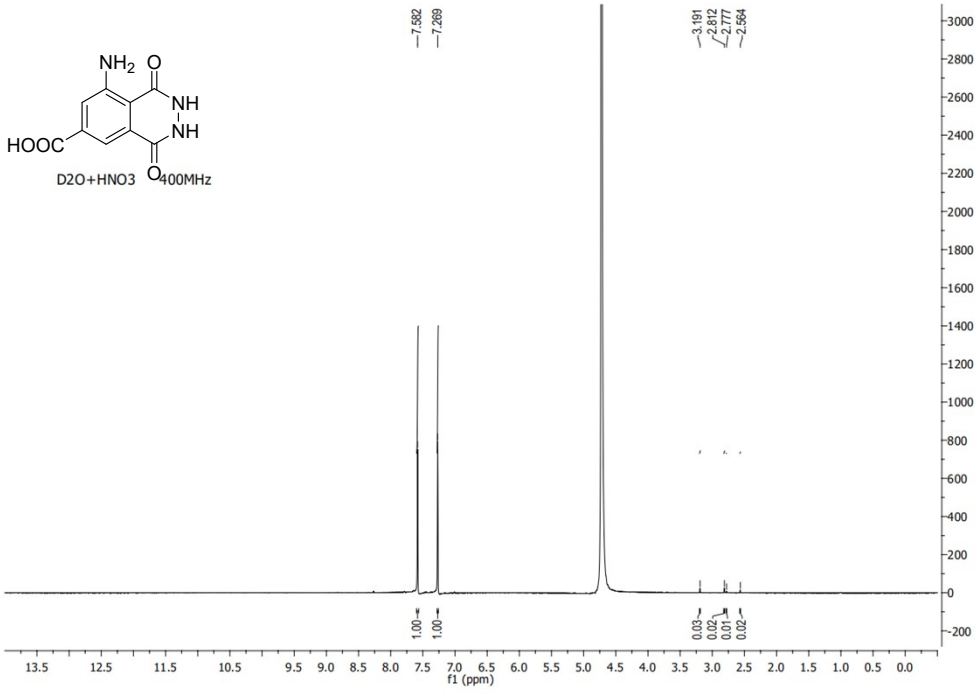


Figure S10: ^{1}H NMR spectrum (D_2O+HNO_3) of compound **C8**

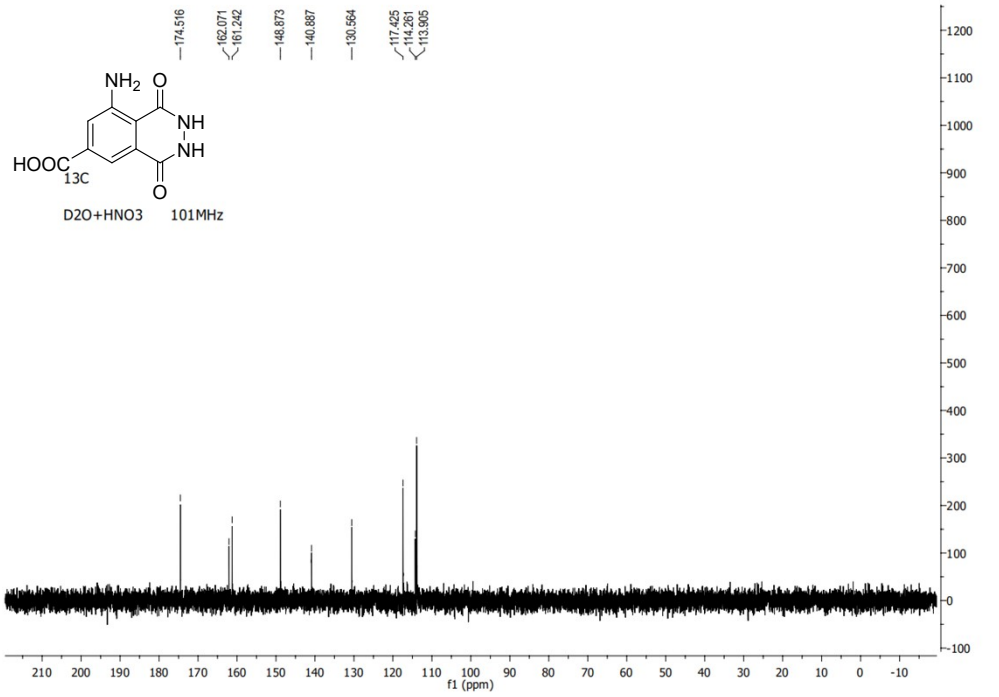


Figure S11: ^{13}C NMR spectrum (D_2O+HNO_3) of compound **C8**

References

[1] Hananya, N.; Shabat, D., A Glowing Trajectory between Bio- and Chemiluminescence: From Luciferin-Based Probes to Triggerable Dioxetanes. *Angewandte Chemie International Edition* **2017**, *56*, 16454-16463.